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1945 VOLUME 39, No. 4

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# The Antibacterial Activity of Simple Derivatives of 2-Aminophenol

By MARY BARBER, *British Postgraduate Medical School, London, W. 12,*
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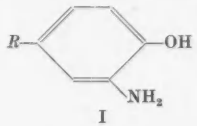
(*Received* 30 *April* 1945)

Discovery of the activity of 2- and 4-aminophenol against several bacterial species, including Gram-negative bacilli, led us to investigate systematically the effect of nuclear substitution on the behaviour of these substances. Preliminary work (Barber & Haslewood, 1944) made us conclude that substituted 4-aminophenols, though possibly active, would be too unstable for therapeutic use, but that a series of 4-substituted 2-aminophenols of the general formula (I) ought to be further investigated. We have now prepared and examined members of this series from $R=H$ to $R=$butyl, and these compounds, with some simple relatives, are listed in Table 1, which also shows approximate antibacterial activities.




I

Antibacterial activity was measured *in vitro* by the methods already described (Barber & Haslewood, 1944). The results shown in Table 1 represent the limiting dilution of each compound causing complete bacteriostasis in nutrient broth, when a small inoculum of less than 500 viable organisms was added.

We conclude that nuclear substitution of 2-aminophenol may provide substances of different antibacterial activity: however, none of the compounds tested by us was definitely more active than 2-aminophenol itself.

The preparation of the substituted 2-aminophenols was effected by hyposulphite reduction of the corresponding nitro-compounds. These were made from the 4-substituted phenols, which were in general the known reference substances in the series. Of these phenols, 4-*iso*butyl phenol does not appear to have been previously crystallized; Niederl, Niederl, Shapiro & McGreal (1937) described it as an oil, whereas our product had m.p. 51–52°.

We made 4-*iso*propyl phenol from cumene (Bert, 1923), but this method, though giving a crystalline product, is unpleasant and the yield is low. It would probably be better to use a modification of the following process which we, in fact, applied to the preparation of 4-*sec*butyl phenol. Racemic 4-*hydroxyphenyl-methyl-ethyl carbinol* was dehydrated by heating its *benzoate* with $MgSO_4$, and the re-

Table 1. *The antibacterial activity of* 4-*substituted* 2-*aminophenols. Limiting dilution causing complete inhibition of growth* (*conditions described in text*)

(Figures indicate ml. containing 1 g. of aminophenol causing inhibition for 1 and 7 days.)

| Compound in series | Substituent in 4 | *E. typhosa* | | *Ps. aeruginosa* | | *Staph. aureus* | |
|---|---|---|---|---|---|---|---|
| | | 1 day | 7 days | 1 day | 7 days | 1 day | 7 days |
| (1) | —H | 16,000–32,000 | 16,000 | 4,000–8,000 | 2,000 | 64,000 | 32,000 |
| (2) | $-CH_3$ | 16,000 | 8,000 | 4,000 | 2,000 | 32,000 | 16,000 |
| (3) | $-CH_2CH_3$ | 16,000 | 8,000 | 4,000 | 4,000 | 32,000 | 16,000 |
| (4) | $-(CH_2)_2CH_3$ | 8,000 | 8,000 | <4,000 | <1,000 | 16,000 | 8,000 |
| (5) | $-CH(CH_3)_2$ | 4,000 | 4,000 | — | — | 16,000 | 16,000 |
| (6) | $-(CH_2)_3CH_3$ | 8,000–16,000 | 8,000–16,000 | 2,000 | <1,000 | 32,000 | 16,000 |
| (7) | $-CH_2CH(CH_3)_2$ | <1,000 | — | <1,000 | — | 64,000 | 16,000 |
| (8) | $-CH(CH_3)CH_2CH_3$ | 8,000 | 8,000 | <1,000 | — | 64,000 | 32,000 |
| (9) | $-C(CH_3)_3$ | 4,000 | 4,000 | <1,000 | — | 16,000 | 8,000 |
| (10) | $-CO.CH_3$ | 4,000 | 4,000 | — | — | 32,000 | 16,000 |
| (11) | $-CO.CH_2.CH_3$ | 2,000 | 2,000 | — | — | 64,000 | 2,000 |
| (12) | $-CO(CH_2)_2CH_3$ | 4,000 | 2,000 | — | — | 4,000 | 4,000 |

*References*

Compound 1: Barber & Haslewood (1944).
„ 2: Heilbron & Bunbury (1943).
„ 3, 4, 6: Baranger (1931)
„ 5, 9: Stevens & Beutel (1941).
Compound 10: Banks & Hamilton (1939).
„ 11: Edkins & Linnell (1936) (hydrochloride).
„ 7, 8, 12: Present work.

sulting *benzoate* of (probably) 4-*hydroxy*-α-*ethylstyrene* was hydrogenated. Hydrolysis of the product gave 4-*sec*butyl phenol in over-all yield (from 4-hydroxy-acetophenone) of about 47%.

In addition to the above carbinol and styrene derivatives, 3-*amino*-4-*hydroxypropiophenone* and 3-*amino*-4-*hydroxybutyrophenone* have not hitherto been described; 2-*amino*-4-*sec*butylphenol and 2-*amino*-4-*iso*butylphenol are also new.

## EXPERIMENTAL

### (A) *Assay of antibacterial activity*

For testing, weighed amounts of aminophenols were dissolved in water, approx. 0·5 N-NaOH, or approx. 0·5 N-$H_2SO_4$. These solutions were then diluted so as to be 0·02 or 0·01 N with respect to NaOH or $H_2SO_4$ and of concentration 0·2 g./100 ml. with respect to the aminophenol. The following solutions of compounds in Table 1 were used: substances (2) and (10) in water; substances (4), (6), (7), (8) and (9) in 0·01 N-$H_2SO_4$; substances (5), (11) and (12) in 0·02 N-NaOH. Compounds (1) and (3) were tested in water, in 0·01 N-$H_2SO_4$, and in 0·02 N-NaOH: no difference in the (considerable) activity of either of these substances could be detected when these different solvents were used.

Serial dilutions of each compound were made in broth consisting of: 'Lab-Lemco' (1 g./100 ml.), peptone (British Drug Houses Ltd., 1 g./100 ml.), NaCl (0·5 g./100 ml.) and *p*-aminobenzoic acid (0·005 g./100 ml.) at pH 7·5. An inoculum of 200–500 bacteria from a diluted 18 hr. culture of the organism to be tested was then added to each tube. Certain variations in the medium did not appreciably alter the results, but a great increase in the number of organisms in the inoculum made a considerable difference (see Barber & Haslewood, 1944). The organisms used were: *Eberthella typhosa*, *Pseudomonas aeruginosa* (*Ps. pyocyanea*), and *Staphylococcus aureus*.

### (B) *Preparation of materials*

Elementary micro-analyses were by Mr A. T. Macdonald, Edinburgh. All melting-points are uncorrected. *Abbreviation:* l.p. = light petroleum (b.p. 40–60°).

4-Hydroxy-acetophenone, -propiophenone, -butyrophenone, and -*iso*butyrophenone were prepared by rearrangement of the appropriate phenol esters under the conditions used by Edkins & Linnell (1936).

4-iso*Butyl-phenol*. 4-Hydroxy-*iso*butyrophenone (5 ml.) was added to the amalgam made by leaving Zn wool (15 g.) for 5 min. with saturated $HgCl_2$ solution (excess) and 10 N-HCl (1 ml.) and then washing it with water. 5 N-HCl (25 ml.) was added at once and the mixture boiled under reflux with more 5 N-HCl (12·5 ml. every 30 min.) for 3 hr. Steam was blown through the product and the oil which came over was extracted with ether. After evaporation of the washed and dried ether, the residue was distilled *in vacuo*. The distillate had b.p. 125–126°/11–12 mm. and formed crystals of m.p. 51–52°. (Found: C, 80·1; H, 9·4%. Calc. for $C_{10}H_{14}O$: C, 80·0; H, 9·3%.)

2-*Amino*-4-iso*butylphenol* (I: $R$ = —$CH_2.CH(CH_3)_2$). A solution of 4-*iso*butylphenol (2 g.) in acetic acid (20 ml.) was treated gradually with 15 ml. of a nitrating solution (8·5 g. $NaNO_3$, 2·3 ml. $H_2SO_4$, 20 ml. water and acetic acid to make the volume 50 ml.). When a few drops of this had been added the mixture was heated on the water-bath until a change of colour showed that reaction had begun; it was then cooled to *c.* 20° and nitration continued at that temperature. After 20 hr. steam was blown through the diluted mixture and the distillate was treated with $Na_2CO_3$ in excess. The filtered solution was acidified with HCl and steam again blown through it. The yellow oil which came over was dissolved, in a volume of *c.* 300 ml., by the addition of 3 g. $Na_2CO_3$. To the boiling solution enough solid $Na_2S_2O_4$ (sodium hyposulphite) was added to discharge the orange colour. The hot liquid was filtered at once, and on cooling at 0°, deposited the aminophenol as leaflets which were collected, washed with cold water and dried at once in the dark *in vacuo* over $H_2SO_4$. Yield, 0·7 g. Recrystallization from l.p.-benzene gave bluish leaflets of m.p. 158–159° (decomp.). (Found: C, 72·7; H, 8·7; N, 8·4%. $C_{10}H_{15}ON$ requires C, 72·7; H, 9·1; N, 8·5%.)

2-*Amino*-4-*sec*-*butylphenol* (I: $R$ = —$CH(CH_3)CH_2CH_3$) was prepared from 4-*sec*butylphenol as above. The product, from l.p.-benzene, had m.p. 127–128° (decomp.). (Found: C, 72·8; H, 8·5; N, 8·5%. $C_{10}H_{15}ON$ requires C, 72·7; H, 9·1; N, 8·5%.)

3-*Amino*-4-*hydroxy-acetophenone* (I: $R$ = —$CO.CH_3$). 3-Nitro-4-hydroxy-acetophenone (0·4 g.), prepared as described by Edkins & Linnell (1936), was dissolved in water (10–15 ml.) with $Na_2CO_3$ (0·8 g.). To the boiling solution $Na_2S_2O_4$ (*c.* 2 g.) was added until the orange colour was discharged. The hot mixture was filtered at once and, at 0°, deposited needles, which were collected, washed with ice-cold water and dried at once in the dark *in vacuo* over $H_2SO_4$. Yield, 0·15 g. Recrystallization from water containing a little $Na_2S_2O_4$ and from l.p.-acetone gave almost white needles whose melting-point after drying was 124–125° (decomp.): these still contained water, and were dried *in vacuo* to constant weight before analysis. (Found: C, 63·9; H, 6·1; N, 9·2%. Calc. for $C_9H_9O_2N$: C, 63·6; H, 6·0; N, 9·3%.) Banks & Hamilton (1939) give the melting-point of this substance as 98°: our preparation had m.p. *c.* 100° before drying.

3-*Amino*-4-*hydroxy-propiophenone* (I: $R$ = —$CO.CH_2CH_3$) was prepared as above. The recrystallized product had m.p. 144–145° (decomp.). (Found: N, 8·4%. $C_9H_{11}O_2N$ requires N, 8·5%.)

3-*Amino*-4-*hydroxy-butyrophenone* (I: $R$ = $CO.(CH_2)_2CH_3$). The corresponding nitro-compound was described by Morgan & Hickinbottom (1921): we made it by the method of Edkins & Linnell (1936). Reduction, as above, gave the aminophenol which, from l.p.-benzene, formed white leaflets, m.p. 115–116° (decomp.). (Found: C, 66·9; H, 7·6; N, 8·05%. $C_{10}H_{13}O_2N$ requires C, 67·1; H, 7·3; N, 7·8%.)

4-*Hydroxyphenyl-methyl-ethyl carbinol*. A Grignard reagent, made from ethyl bromide (16·8 g.), ether (100 ml.), and Mg (4 g.), was cooled with water, while a solution of 4-hydroxy-acetophenone (6·8 g.) in dry ether (*c.* 150 ml.) was added gradually down a reflux condenser. The mixture was thoroughly shaken, with cooling, then boiled for 30 min. under reflux and left at *c.* 20° for 16 hr. After careful decomposition with water, $NH_4Cl$ (10 g.) and 5 N-$H_2SO_4$, the product was separated with the ethereal layer, which was washed with water, dried ($Na_2SO_4$) and evaporated. The residue crystallized with l.p. giving colourless prisms of

m.p. 118–121°. Yield, 6·6 g. A sample, after sublimation at 105°/0·2 mm., had m.p. 120–121°. (Found: C, 72·1; H, 8·8%. $C_{10}H_{14}O_2$ requires C, 72·3; H, 8·4%.)

The above substance (12 g.), dissolved in 5 N-NaOH (120 ml.), was shaken with benzoyl chloride (25 ml. at once, and 10 ml. after 20 hr.). After 24 hr. the precipitated solid was collected, well washed with water, and dissolved in ether. The ether was dried ($Na_2SO_4$), filtered and evaporated. The residue, with l.p. formed crystals (17 g.) of m.p. 77–81°. A sample, after sublimation at 145°/0·05–0·1 mm., separated from l.p.-benzene as large colourless prisms, m.p. 83–84°, of *4-benzoyloxyphenyl-methyl-ethyl carbinol*. (Found: C, 75·7; H, 6·3%. $C_{17}H_{18}O_2$ requires C, 75·5; H, 6·7%.)

*(Probably) 4-Benzoyloxy-α-ethyl-styrene.* An intimate mixture of the above benzoate (3 g.) with freshly dried finely powdered anhydrous $MgSO_4$ (12 g.) was heated at 160–170° for 1 hr. Dilution of the cooled product gave a water-insoluble solid, which was collected, washed, and crystallized from methanol, giving almost colourless needles (1·75 g.); m.p. 111–112°. (Found: C, 80·7; H, 6·7%. $C_{17}H_{16}O_2$ requires C, 80·9; H, 6·4%.)

*4-sec.Butyl-phenol.* The above compound (2 g.) in benzene (50 ml.) was shaken with $H_2$ and Pd-black (0·5 g.) until no more $H_2$ was absorbed. The product, obtained by evaporation of the filtered solution, was an oil at 20°. It was boiled under reflux with N-NaOH (40 ml. in approx. 30% v/v ethanol-water) for 3 hr. Ethanol was then removed in steam, and the alkaline residue was acidified with HCl. The oily phenol was separated by steam distillation and formed an aqueous suspension, which crystallized at 0°. Yield, 1·3 g., m.p. 51–53°; Reilly & Hickinbottom (1920) record m.p. 59°.

## SUMMARY

1. A series of substituted 2-aminophenols has been tested *in vitro* for antibacterial activity.

2. It is concluded that such activity is not increased but may be decreased by simple nuclear substitution of 2-aminophenol.

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# A Metabolite of 2-Acetamidofluorene*

By F. BIELSCHOWSKY, *Department of Pathology and Cancer Research Laboratories, University of Sheffield*

(*Received* 15 *May* 1945)

Investigation of the fate of carcinogenic and related non-carcinogenic hydrocarbons in animals was begun by Boyland & Levi (1935) and by Chalmers & Peacock (1936), a few years after the discovery of the first carcinogenic compound of known structure (Kennaway, 1930). The presence of phenolic derivatives of carcinogenic hydrocarbons in the excreta of rabbits, rats and mice was recognized at an early stage of these studies, and some of the metabolites were later isolated and identified. Fieser (1941), reviewing the results of this line of cancer research, came to the conclusion that: 'biological hydroxylation represents an avenue for the elimination of carcinogens in a detoxified form', and that 'the reaction of detoxication, if indeed it may be so regarded, involves an attack of the molecule at some site other than the centre of special susceptibility to substitutions'. Relevant data obtained since 1941 conform in the main with Fieser's views. Berenblum & Schoental (1943) called attention to the fact that 'with benzanthracene and dibenzanthracene, the positions in the molecule metabolically attacked are also those where sulphonation occurs in vitro, provided the most reactive positions are blocked'.

It seemed of interest to find whether these rules applied to 2-acetamidofluorene,* one of the most potent of the carcinogens which produce distant tumours (Wilson, DeEd & Cox, 1941; Bielschowsky, 1944; Armstrong & Bonser, 1944). Wilson *et al.* (1941) observed that the fur of the experimental rats, and the white pine shavings on which they

* Previous authors have described this compound as 2-acetylaminofluorene. It is here described as 2-acetamidofluorene, in conformity with the rules of organic nomenclature adopted.

were kept, were stained when higher dosages of acetamidofluorene were fed. They suspected that 2-aminofluorene, excreted with the urine, was responsible for this bright orange colour, but were unable to isolate aminofluorene from the urine of rats and rabbits receiving acetamidofluorene by mouth; nor could they obtain a derivative of quinonoid structure.

## RESULTS

Our first efforts were directed to obtaining metabolites from the urine of rats receiving 2-acetamidofluorene, since the observations of Wilson *et al.* (1941) had made it highly probable that derivatives of the carcinogen are excreted by the kidneys. One metabolite could be isolated without great difficulty. The compound, a substance of phenolic character, crystallized in colourless needles, m.p. 232°. The analysis was in agreement with the formula $C_{15}H_{13}O_2N$. The presence of an acetyl group was established, thus indicating that the substance is a monohydroxy derivative of acetamidofluorene. The melting-point and absorption spectrum of the isolated compound were found to be identical with the melting-point and absorption spectrum of synthetic 2-acetamido-7-hydroxyfluorene. The amount of the metabolite which could be isolated corresponded to about 5–8 % of the administered carcinogen.

2-Acetamido-7-hydroxyfluorene gives a very sensitive colour reaction with nitrite, which can be applied to ether extracts of urine. By this method it was found that the excretion of 2-acetamido-7-hydroxyfluorene ceases 2–3 days after withdrawal of the drug from the diet, independently of the duration of administration.

2-Acetamido-7-hydroxyfluorene is not the only metabolite of 2-acetamidofluorene excreted with the urine, but it seems to be the derivative which is excreted in largest quantities, and others have not yet been identified.

## EXPERIMENTAL

Albino rats received a daily dosage of 4–6 mg. of 2-acetamidofluorene with their diet (skimmed milk-bread). The animals were kept in metabolism cages. The collected urine was stored in a refrigerator, preserved with chloroform, until 1 l. was available.

All melting-points are uncorrected. Analyses were carried out by Dr G. Weiler (Oxford).

*Isolation of 2-acetamido-7-hydroxyfluorene.* The urine (1000 ml.), which was alkaline to litmus, was extracted five times with 250 ml. of ether. Ethanol (10 ml.) was added to break the emulsions, but sometimes it was necessary to centrifuge in order to obtain a satisfactory separation. The combined ether extracts were dried over sodium sulphate and concentrated to about 250 ml. The ether was washed with dilute HCl and water, further concentrated to a volume of about 10–15 ml. and poured into a flask containing 50 ml. of water. After 2 days' standing in a refrigerator, crystallization started at the interface of the ether-water layers and increased slowly during the next few days. The crystals were collected and washed with ice-cold ether. The substance so obtained was still very impure and melted between 190 and 200°.

For purification 100 mg. of these crystals were boiled under reflux with 10 ml. of anhydrous ether (peroxide-free). The ether extracted most of the coloured impurities. The undissolved portion (80 mg.) was recrystallized first from 50 % (v/v) acetic acid with the addition of charcoal and then from aqueous ethanol, m.p. 232°. (Found: C, 75·03; H, 5·69; N, 5·95 %. $C_{15}H_{13}O_2N$ requires C, 75·28; H, 5·48; N, 5·86 %.)

*Synthesis of 2-acetamido-7-hydroxyfluorene.* 2-Amino-7-nitrofluorene (7-amino-2-nitrofluorene) was prepared according to the method of Diels, Schill & Tolson (1902) and diazotized as follows: 2 g. of this substance were dissolved in 500 ml. of boiling 2 N-$H_2SO_4$, the solution was cooled quickly to about 40° and 1 g. of $NaNO_2$ added with stirring, the temperature being maintained at 40–45°. The reaction mixture was filtered, urea added and the solution boiled under reflux for 90 min. 7-Hydroxy-2-nitrofluorene crystallized. The product was collected, washed with water and dried. After recrystallization from toluene it had m.p. 250°. (Found: N, 6·07 %. $C_{13}H_9O_3N$ requires N, 6·17 %.)

2-Amino-7-hydroxyfluorene was obtained from the crude nitro-compound. 1 g. nitro-compound was mixed with 5 g. zinc dust and 1 g. anhydrous $CaCl_2$, 100 ml. of 80 % (v/v) ethanol were added and the mixture was boiled under reflux for 60 min. on a water-bath. The dark yellow solution turned colourless. The zinc dust was filtered off and on addition of water to the hot filtrate a slightly coloured, crystalline precipitate was obtained, which was collected after standing in a refrigerator for several hours. The precipitate was dried *in vacuo*. After recrystallization from aqueous ethanol it melted with decomposition at 271°. (Found: N, 7·18 %. $C_{13}H_{11}ON$ requires N, 7·11 %.)

2-Acetamido-7-hydroxyfluorene. The crude amino-compound was dissolved in 200 ml. of water by the addition of 2 ml. of conc. HCl. The solution was filtered from a small amount of a coloured impurity and 1 g. crystalline sodium acetate and excess acetic anhydride were added. The solution was stirred for 2 hr. in an ice-bath. After a few minutes the acetyl compound began to crystallize. The product was collected and recrystallized first from aqueous acetic acid and then from aqueous ethanol, m.p. 232°. (Found: C, 75·18; H, 5·49; N, 5·85 %. $C_{15}H_{13}O_2N$ requires C, 75·28; H, 5·48; N, 5·86 %.)

*Comparison of the isolated and synthetic compounds.* Both substances had the same melting-point and no depression was observed in a mixture of the two. They showed no differences in solubility and both gave the same colour reaction with nitrite. Dr F. B. Strauss (Oxford) compared the absorption spectra of the isolated and synthesized compounds and gave the following opinion: 'both spectra are approximately identical; moreover the wave-lengths of maximum absorption as well as the molar extinction coefficients of maximum absorption are identical within the limits of experimental error.'

*The colour reaction.* 2-Acetamido-7-hydroxyfluorene (1 mg.) was dissolved in 5 ml. glacial acetic acid. 0·5 ml. of this solution was diluted with an equal volume of water, and a drop of 2N-HCl and four drops of a freshly prepared 0·1% solution of sodium nitrite were added. A deep purple colour developed. The dye could be extracted with amyl alcohol. Amounts as small as 20 $\mu$g. are still detectable with this reaction.

The ether extract of the urine of rats receiving a diet containing 2-acetamidofluorene showed a strong nitrite reaction: 250 ml. of urine were extracted as described above, the ether was dried over sodium sulphate, evaporated and the residue dissolved in 5 ml. of glacial acetic acid. Of this extract 0·1 ml. still showed a positive nitrite reaction. When collection of the urine of rats was commenced 48 hr. after the withdrawal of the drug from the diet and the urine was treated in the same way, the extracts did not show a positive test, even when amounts corresponding to 50 ml. of urine were taken.

## DISCUSSION

The isolation of 2-acetamido-7-hydroxyfluorene from the urine of rats receiving 2-acetamidofluorene suggests that the observation of Berenblum & Schoental (1943) is not confined to benzanthracene and dibenzanthracene. When 2-aminofluorene is treated with sulphuric acid 2-amino-7-fluorenesulphonic acid is formed (Courtot, 1930). The biological hydroxylation has thus taken place in the same position as that in which sulphonation occurs *in vitro*.

## SUMMARY

One metabolite of 2-acetamidofluorene has been isolated from the urine of rats, and has been identified as 2-acetamido-7-hydroxyfluorene.

This work was carried out under the aegis of the Yorkshire Branch of the British Empire Cancer Campaign.

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# Purification and Properties of Cytochrome *c*

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(*Received* 16 *May* 1945)

It was previously shown that cytochrome *c* can be extracted in a soluble form from baker's yeast (Keilin, 1930) or from heart muscle of horse or ox (Theorell, 1935; Keilin & Hartree, 1937), and that fractionation of the cytochrome *c* from heart muscle gives rise to a preparation containing 0·34% Fe. A very simple method of preparation of cytochrome *c* of this purity was described (Keilin & Hartree, 1937), and this method has been generally adopted by other workers in their studies of this substance.

Attempts to increase the iron content above 0·34% were unsuccessful, and the protein appeared to be homogeneous in cataphoretic experiments. While determining the isoelectric point of cytochrome *c* in the Tiselius apparatus, Theorell & Åkesson (1939) noticed that at pH slightly above the isoelectric point cytochrome *c* containing 0·34% Fe no longer migrated as a homogeneous protein. The isoelectric point of cytochrome *c* was given as 10·65 at 0°, and at pH 10·68 the red cytochrome *c* moved slowly towards the anode while a colourless boundary moved more rapidly (Theorell & Åkesson, 1941). By the use of a large Tiselius (1938) apparatus and continuous electrophoresis of 9–10 days' duration, Theorell & Åkesson were able to separate the colourless fraction from a considerable quantity of

cytochrome *c* containing 0·258–0·31 % Fe and to obtain material with an iron content of 0·43 %. This fraction appeared to be homogeneous by cataphoresis at all pH values. Although the amino-acid analyses and some physical properties of this purified cytochrome were recorded by Theorell & Åkesson (1941), the most fundamental property of cytochrome, viz. its activity as a biological oxidation catalyst, was not examined by them.

The object of this investigation was to compare the catalytic activity of this new preparation (0·43 % Fe) with that of our usual purified preparation (0·34 % Fe), especially as it was suggested by these workers that only the electrophoretically purified cytochrome *c* should be used in enzyme studies. For this purpose cytochrome *c* containing 0·43 % Fe was prepared by a new method not requiring the large-scale Tiselius apparatus, which is not usually available to workers engaged in the study of intracellular catalysts.

After several years' experience in the preparation of cytochrome *c* containing 0·34 % Fe* we have been led to introduce a few minor modifications in our original method of preparation, which will therefore be redescribed.

## METHODS

*Estimation of iron.* 1–3 ml. cytochrome solution is combusted in a Kjeldahl flask with $H_2SO_4$ plus the occasional addition of a drop of $KClO_3$ solution. The acid is evaporated almost to dryness, treated with 5 ml. distilled water, 5 mg. $\alpha\alpha'$-dipyridyl and 1 g. $Na_2SO_3.7H_2O$. The pink colour develops on standing 30 min. The solution is diluted to 10 ml. and either (1) compared in a colorimeter with a standard iron-dipyridyl solution, or (2) estimated by measuring the extinction coefficient at 521 mμ., the wave-length of maximum absorption. By working with known amounts of iron it was found that the iron concentration was related to the extinction coefficient by the equation $c = 0{\cdot}00635K_{521}$, where $c$ = concentration of Fe in mg./ml. and $K = 1/d \log_{10} I_0/I$. The second method was found to be the more reliable one.

*Dry weight* of cytochrome in presence of NaCl or KCl was estimated by evaporating the solution at 100° to constant weight in a platinum crucible and measuring the loss in weight after ignition for 1 hr. at 500°. Dry weight of cytochrome = loss in weight + weight of $Fe_2O_3$ or loss in weight + 1·4 × (weight of Fe), the latter having already been determined. The second term ($Fe_2O_3$) is almost within the experimental error; hence the actual constitution of the oxide is unimportant although the brown colour indicates that it is $Fe_2O_3$. In many cases iron was estimated directly in the residue from the incineration, which dissolved readily in hot dilute $H_2SO_4$.

The reliability of this method was confirmed on preparations the dry weight of which was subsequently redetermined after dialysis against distilled water until salt-free.

* It was stated by Theorell & Åkesson (1941) that our method normally yields material containing less iron than 0·34 %, though in our hands the method, even without the newer modifications, almost always yields cytochrome with an iron content of 0·34 %.

*Preparation of cytochrome c from horse heart*

(*a*) *Preparation containing* 0·34 % Fe. A horse heart is cut open as soon as possible after collection from the slaughter-house and the blood allowed to drain out. The muscle is freed from fat and ligaments and finely minced. To the minced muscle is added 0·145 N-trichloroacetic acid at the rate of 1 l./kg. mince. The acid is added in three or four lots with adequate mixing after each addition. The trichloroacetic acid is prepared by diluting a 20 % solution, the exact strength of which has been determined by titration with N-NaOH, using phenolphthalein as indicator. After standing 3–4 hr. the acid mixture is squeezed out in a press and the cloudy fluid brought to pH 7·3 with 10 % NaOH. Ammonium sulphate is now added at the rate of 500 g./l., and when it is dissolved the mixture is placed on large fluted filter papers and left overnight to drain completely. The clear pink filtrate, which is free from all pigments except cytochrome *c*, is treated again with $(NH_4)_2SO_4$ at the rate of 50 g./l. and left overnight at 0–5°. If any precipitate forms it is filtered off and the cytochrome in the cold filtrate is precipitated by the addition of 20 % trichloroacetic acid (25 ml./l.). Within a few minutes the cytochrome aggregates and is centrifuged down. The red precipitate is suspended in about 300 ml. saturated $(NH_4)_2SO_4$ and centrifuged again. It is now suspended in the minimum amount of distilled water and transferred to a cellophan sac in which it is dialyzed against 0·5 % NaCl until free from $(NH_4)_2SO_4$. The suspension is shaken with a few drops of chloroform and the precipitate is filtered off. 30–40 ml. of a dark red solution of cytochrome *c* are obtained, containing about 1 % protein. The iron content is usually 0·34 %, but in cases where it is lower it can be brought up to this value by diluting the solution to 500–1000 ml. and repeating the ammonium sulphate-trichloroacetic acid precipitation. While saturated with chloroform the solution of cytochrome thus obtained keeps indefinitely at 5° in a well-stoppered bottle.

Some workers in this field have preferred to dialyze cytochrome against distilled water rather than against dilute NaCl. In our experience the cytochrome in complete absence of salts tends to become absorbed by the cellophan. The estimation of dry weight of protein in presence of salts is described above.

(*b*) *Preparation containing* 0·43 % Fe. 120 ml. of the above solution of cytochrome *c* (0·34 % Fe) are treated with $(NH_4)_2SO_4$ until a small quantity of the latter remains undissolved. 20 ml. ammonia, sp.gr. 0·880, are added drop by drop to give pH 10 (10·06 as measured with a glass electrode) and the mixture left overnight at 5°. Next day the precipitate *A* is removed and the filtrate is freed from

Table 1. *Analyses of various cytochrome c fractions*

(Average for $B_1$, $B_2$ and $B_3$ = 0·426 % Fe)

| Preparation | Dry wt. (mg./ml.) | Fe (mg./ml.) | Fe (%) | Total vol. (ml.) | Yield in terms of Fe (%) |
|---|---|---|---|---|---|
| Original solution cytochrome *c* | 7·2 | 0·0247 | 0·34 | 120 | (100) |
| Solution $B_1$ | 9·39 | 0·0402 | $0·42_9$ | 60 | 81 |
| Solution $A_1$ | 15·25 | 0·030 | 0·20 | 10·5 | 11 |
| Fractionation of 40 ml. $B_1$: $B_2$ | 6·24 | 0·0265 | $0·42_5$ | 30 | 49 |
| $B_3$ | 3·04 | 0·0129 | $0·42_4$ | 9 | 7 |
| Fractionation of 9·5 ml. $A_1$: $A_2$ | 2·89 | 0·0117 | $0·40_5$ | 10 | 40 |

$(NH_4)_2SO_4$ by dialysis in a cellophan tube for 3 hr. against running tap water and finally against 0·5 % KCl. The resulting solution (250 ml.) is reduced in bulk to about 50 ml. by suspending the cellophan tube in a current of air. After a further dialysis against KCl the solution $B_1$ (60 ml.) is collected. The cytochrome of this fraction contains 0·43 % Fe (Table 1).

The homogeneity of this fraction was tested in three ways:

(1) When tested in the Tiselius apparatus it showed only one moving boundary, both at pH 7·3 and at pH 10·7.

(2) A diffusion experiment at pH 7·3 also indicated a pure substance.

(3) Fractionation of the solution by absorption on and elution from calcium phosphate gel yielded cytochrome fractions, $B_2$ and $B_3$, with unchanged iron contents (Table 1).

The precipitate $A$ is washed with water containing $(NH_4)_2SO_4$ and $NH_3$ in concentrations equal to those added to the original cytochrome solution. When the washings become almost colourless the precipitate is dissolved in a little water and dialyzed against tap water and finally distilled water. A small quantity of denatured protein is filtered off to give solution $A_1$ (10·5 ml.). This fraction contains 0·20 % Fe. It is decidedly more brown than $B_1$ or the original solution and contains some modified pigment.

Attempts to isolate an appreciable amount of the colourless fraction from $A_1$ were not successful. 90 % saturation with $(NH_4)_2SO_4$ yielded a pale pink-brown precipitate which could not be sharply separated into coloured and colourless fractions. After removal of the precipitate the cytochrome still in solution was thrown down by saturating with $(NH_4)_2SO_4$ at 60°. It contained 0·40 % Fe ($A_2$) and would therefore appear to consist essentially of the cytochrome containing 0·43 % Fe.

### *Catalytic activity of the two cytochrome preparations*

The catalytic activities of the original cytochrome *c* (0·34 % Fe) and of the preparation $B_2$ (0·43 % Fe) were compared in two systems obtained from a kidney preparation: (1) the system oxidizing ascorbic acid and (2) the system oxidizing succinic acid. Kidney preparation, which we have previously described (Keilin & Hartree, 1940), has the advantage in such experiments of being deficient in

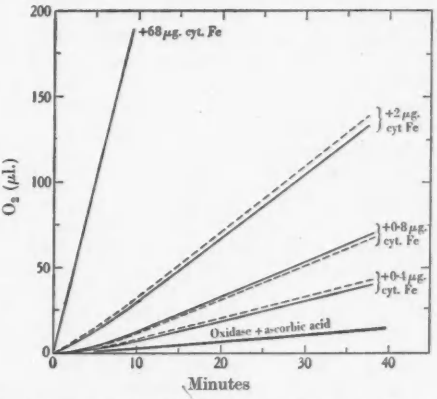


Fig. 1. Comparison of the catalytic effects of two cytochrome *c* fractions containing 0·34 % Fe (full lines) and 0·43 % Fe (dotted lines) in the system cytochrome oxidase (0·1 ml. sheep kidney extract) + ascorbic acid (15 mg.). Temp. = 39°; pH = 7·3.

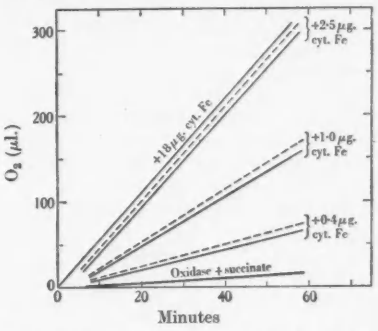


Fig. 2. Comparison of the catalytic effects of two cytochrome *c* fractions in oxidation of succinate (0·04 M) by kidney extract (0·25 ml.). Experimental conditions as for Fig. 1.

cytochrome *c*, hence the oxidation of ascorbic acid, succinic acid, and other substances is very slow in

the absence of added cytochrome *c*. Experiments were carried out at 39° and pH 7·3 in Barcroft differential manometers under the usual conditions. The components of the systems investigated are:

(1) Cytochrome oxidase + cytochrome *c* + ascorbic acid.

(2) Cytochrome oxidase + cytochrome *c* + succinic dehydrogenase + succinic acid.

Cytochrome oxidase and succinic dehydrogenase are represented by the kidney preparation. When the enzymes and the substrate are in excess the $O_2$ uptakes of the systems serve as measures of the catalytic power of cytochrome *c*. The results of these experiments, which are shown in Figs. 1 and 2, clearly demonstrate that the catalytic activities of the iron in the two cytochrome preparations are identical, and that both preparations can equally well be used in work with enzymes.

The comparative study of other properties of these two preparations, namely, their absolute absorption spectra and their reactions, at varying pH values with $O_2$ and CO, did not reveal any noticeable differences.

## DISCUSSION

The increase in the iron content of cytochrome *c* from 0·34 to 0·43 %, which can be obtained either by a prolonged cataphoresis at pH > 10·65 (Theorell & Åkesson, 1941), or by fractionation at pH 10 as described above, indicates a proportional increase in the purity of the compound. This has been corroborated by studying the catalytic activities per atom of iron of these two preparations, which were found to be equal. Hence removal of about 20 % of the protein of the molecule has not affected the properties of the prosthetic group. On the other hand, this final step of purification cannot be considered as a removal of some indifferent impurities which arbitrarily become associated with cytochrome *c* during its preparation. It is conceivable that the protein fraction separated at this very alkaline pH forms part of the original intracellular cytochrome *c*. In fact, cytochrome preparations containing 0·34 % Fe were obtained by two different methods (Theorell, 1935; Keilin & Hartree, 1937). Moreover, these preparations behave in every respect as homogeneous proteins at pH below 10, and only at pH > 10·65 in electrophoretic experiments does the protein of cytochrome (0·34 % Fe) dissociate into two fractions: a colourless fraction representing about 20 % of the total solids, and the remaining red cytochrome *c* containing 0·43 % Fe. It seems unlikely that cytochrome *c* obtained by different methods should combine in the same proportions with accidental impurities. The dissociation of such a complex at a very alkaline pH resembles the dissociation of other apparently pure proteins such as haemocyanin at extreme pH (Redfield, 1934). It must also be remembered that whereas cytochrome containing 0·34 % Fe corresponds to a molecular weight of 16,500 which has been found for several proteins (Svedberg & Pedersen, 1940), the iron content of 0·43 % indicates a molecular weight of about 13,000 which is unprecedented among proteins.

As the two cytochrome preparations show no marked differences in their properties, both can be used equally well in experiments with enzymes or in the reconstruction of certain intracellular catalytic systems. It is conceivable that the colourless protein forms an integral portion of the intracellular cytochrome molecule, playing some part in its catalytic activity within cells, where this component exhibits a much greater efficiency than it does in any experiments *in vitro* (Keilin & Hartree, 1940).

## SUMMARY

1. By slight modifications of our original method the preparation of cytochrome *c* containing 0·34 % Fe has been simplified.

2. By submitting this compound to electrophoresis Theorell & Åkesson (1941) found that below its isoelectric point (pH 10·65) it appears to be homogeneous, whereas at a higher pH (10·68) it dissociates into a colourless protein (20 % of the total) and red cytochrome *c* containing 0·42–0·43 % Fe. This raising of the iron content of cytochrome *c* has been confirmed by fractional precipitation of the preparation (0·34 % Fe) at pH 10·0, i.e. a method not requiring specialized apparatus.

3. Both cytochrome preparations containing 0·34 and 0·43 % Fe show the same catalytic activity per atom of iron; hence the preparation containing 0·34 % Fe is suitable for experiments with enzymes and the reconstruction of intracellular oxidizing systems.

4. The significance of this final step of purification of cytochrome *c* is discussed.

The authors wish to thank Dr H. Gutfreund for testing various cytochrome fractions in the Tiselius cataphoresis apparatus and Dr R. Markham for confirming the homogeneity of our purest preparation by diffusion measurements.

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# Properties of Catalase. Catalysis of Coupled Oxidation of Alcohols

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(*Received* 16 *May* 1945)

It was previously shown (Keilin & Hartree, 1936) that on addition of ethanol and catalase to a primary oxidation system, such as xanthine oxidase and hypoxanthine, the rate of oxygen uptake is markedly increased and the final uptake is doubled. Similar results were obtained using other oxidizing enzymes, e.g. uricase and *d*-amino-acid oxidase and their respective substrates, in other words systems which react directly with molecular $O_2$ and reduce it to $H_2O_2$. The increased $O_2$ uptake was shown to be due to the secondary oxidation of ethanol to acetaldehyde by $H_2O_2$ formed in the primary oxidation reaction. It was a matter for some surprise to find that this secondary oxidation of ethanol took place only in presence of catalase, which catalyzes this reaction instead of preventing it by the usual catalatic decomposition of $H_2O_2$ into $O_2$ and water. Since $H_2O_2$ formed in the primary oxidation reaction could be replaced by barium or cerium peroxide, but not by a solution of free $H_2O_2$, it appeared that the $H_2O_2$ oxidizing ethanol was in a 'nascent state'.

In this paper we propose to deal with three aspects of this reaction: (1) the catalysis of the reaction by pure catalase in order to eliminate the possible presence of other catalytic systems reacting with alcohols, (2) the comparison of the rate of oxidation of different alcohols, and (3) the nature of the peroxide required for this oxidation reaction. This study will be concluded by a critical examination of the possible function of catalase.

## METHODS

*Oxidation systems.* We have used three primary oxidation systems which reduce $O_2$ to $H_2O_2$:

(1) *Xanthine oxidase* in the form of a crude caseinogen preparation was obtained by precipitating milk by half saturation with $(NH_4)_2SO_4$, drying the precipitate between filter papers and defatting it with pure ether. Its substrate hypoxanthine is oxidized via xanthine to uric acid.

(2) d-*Amino-acid oxidase* was used in the crude form as an acetone dried mince of pig's kidney and also in a pure form prepared according to Negelein & Brömel (1939). Its substrate *d*(−)-alanine is oxidized to pyruvic acid.

(3) *Glucose oxidase* was obtained from *Penicillium notatum* in a highly purified state (notatin) and was kindly supplied by Prof. Raistrick and Dr Birkinshaw. Its substrate glucose is oxidized to gluconic acid.

*Catalase* used in these experiments was either a highly purified enzyme preparation obtained from horse liver (Keilin & Hartree, 1945) or a crystalline ox-liver catalase prepared according to Sumner & Dounce (1937).

The experiments were carried out as previously described (Keilin & Hartree, 1936) in Barcroft manometers at 39° in 0·1 M-phosphate buffer of pH 7·8 for xanthine oxidase, 7·3 for crude amino-acid oxidase, and 6·0 for glucose oxidase. For the purified amino-acid oxidase a pyrophosphate buffer was used (0·07 M, pH 8·3).

*Estimation of acetaldehyde.* The method used was based on the Schiff reaction. 1–5 ml. of the solution to be analyzed containing $> 100\mu g.$ acetaldehyde were added to 5 ml. 5 N-$H_2SO_4$ in a boiling tube. The tube was warmed to 80° and a slow current of air, purified by passage through charcoal, was passed through the solution at a rate of 100–150 ml./min. The air-stream was then bubbled through 1 ml. of Schiff's reagent in a small tube 6 × 1 cm. kept at 0°. The entire apparatus was made up with ground-glass joints. After 10 min. aeration the reagent was transferred to another small tube and, after a further 20 min. at 0°, was compared with a series of freshly made standards. $5\mu g.$ acetaldehyde are just detectable. The reagent is prepared from a 0·1% solution of pure fuchsin treated with $SO_2$ until almost colourless: on short standing the remaining colour disappears. 20 min. aeration under the standard conditions should give no coloration. The reagent should be decolorized freshly each day. The error for a single determination is of the order of 10%, but the method has the advantage of simplicity.

## EXPERIMENTS

### *Oxygen uptake by the primary oxidation system*

Since the above oxidizing systems reduce $O_2$ to $H_2O_2$, the $O_2$ uptake should be double the amount calculated for the oxidation of the substrate. However, this does not apply generally to all three systems. Thus in the xanthine oxidase system the total $O_2$ uptake differs according to the experimental conditions. It may be below the theoretical amount in the case of a purified enzyme preparation which is easily inactivated by the peroxide formed in the reaction (Dixon, 1925). It may reach the theoretical amount in the case of the caseinogen preparation which is more stable, the $H_2O_2$ formed during the oxidation of the substrate acting as a hydrogen acceptor for the oxidation of more of the same substrate (Dixon, 1926). It may be above the theoretical amount owing to a secondary oxidation of impurities by means of $H_2O_2$ and peroxidase present in the crude preparations.

With a crude preparation of *d*-amino-acid oxidase the total $O_2$ uptake usually reaches the theoretical amount, which is due to the presence of a sufficient concentration of catalase for the decomposition of $H_2O_2$. In a purified catalase-free preparation the

$O_2$ uptake is double the theoretical amount (see below).

In the glucose oxidase system the total $O_2$ uptake is double the theoretical amount, owing to the complete absence of catalase and to the resistance of the enzyme to $H_2O_2$.

In all the control experiments referred to in this paper, the theoretical $O_2$ uptake with any of the three oxidizing enzyme systems was invariably obtained by carrying out experiments in presence of pure catalase.

*Oxidation of alcohols in presence of the xanthine oxidase system plus catalase*

We repeated the experiments which we have previously described, this time using purified catalase.

The experiments were carried out in four Barcroft manometers, the right-hand flasks of which received about 100 mg. oxidase preparation (caseinogen) and a dangling tube containing 1 mg. hypoxanthine in 0·3 ml. dilute NaOH. In addition to oxidase the flasks of the second, third and fourth manometers received respectively catalase (≡0·016 mg. haematin), ethanol (10 mg.) and catalase plus ethanol. The solution in the flasks was made to 3 ml. with 0·1 M-phosphate buffer pH 7·8. In other series of similar experiments the central tubes of the flasks received 0·3 ml. 10% KOH and a roll of filter paper.

A typical result of such experiments (Fig. 1) shows that the $H_2O_2$ formed in the primary oxidation reaction is utilized by purified catalase for the

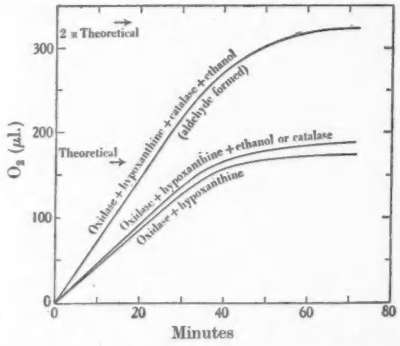


Fig. 1. Coupled oxidation of ethanol by the xanthine oxidase system in presence of purified horse-liver catalase. Xanthine oxidase = 100 mg. caseinogen preparation of milk, 1 mg. hypoxanthine (≡167 µl. $O_2$), 10 mg. ethanol, 0·3 ml. catalase. Temp. 39°; pH 7·8. Similar results were obtained using recrystallized ox-liver catalase.

oxidation of ethanol to acetaldehyde. The formation of aldehyde is easily recognized not only by smell but also by yellowing of the potash papers due to resin formation.

*Cyclic oxidation of ethanol to aldehyde*

We have shown previously (Keilin & Hartree, 1936) that when the primary oxidizing system is composed of xanthine oxidase and aldehyde (i.e. the Schardinger system), the $H_2O_2$ formed, being used by catalase for the oxidation of ethanol to acetaldehyde, increases the supply of substrate for the primary oxidation reaction. The reaction will therefore proceed as long as ethanol is present and the enzymes involved are still active. This reaction, in which the product of the secondary oxidation

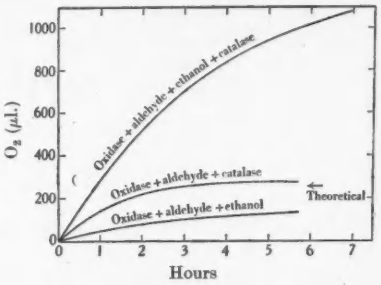


Fig. 2. Cyclic oxidation of ethanol by the Schardinger system and purified catalase: 300 mg. caseinogen preparation of milk, 1 mg. acetaldehyde, 5 mg. ethanol, 0·3 ml. catalase. Experimental conditions as in Fig. 1.

supplies the substrate for the primary oxidation, was described by us as a 'cyclic oxidation'. We have been able to obtain this reaction with a highly purified horse-liver catalase (Fig. 2) as well as with the crystalline catalase prepared from ox liver.

*Coupled oxidation of ethanol by purified d-amino-acid oxidase*

It was mentioned above that when a purified catalase-free preparation of amino-acid oxidase catalyzes the oxidation of *d*(−)-alanine the $O_2$ uptake is double the theoretical amount calculated for oxidation of the substrate. This is due, as was shown by Negelein & Brömel (1939), to a spontaneous reaction between the two products of this oxidation, namely, $H_2O_2$ and pyruvate, which yield acetic acid and $CO_2$. In this case the reactions proceed as follows:

$$CH_3.CH(NH_2).COOH + O_2 + H_2O = CH_3.CO.COOH + NH_3 + H_2O_2$$

$$CH_3.CO.COOH + H_2O_2 = CH_3.COOH + CO_2 + H_2O$$

$$CH_3.CH(NH_2).COOH + O_2 = CH_3.COOH + NH_3 + CO_2$$

Following the description given by these workers, we prepared their enzyme fraction *D*, which is free

Table 1. *Effect of catalase and ethanol on the system purified amino-acid oxidase + d(−)-alanine*

*d*-Amino-acid oxidase is composed of the enzyme protein + alloxazine adenine dinucleotide. The oxidation of 1·5 mg. *d*(−)-alanine (or 3 mg. *dl*-alanine) to pyruvate requires 188 $\mu$l. $O_2$: $CH_3CH(NH_2)COOH + \frac{1}{2}O_2 = CH_3.CO.COOH + NH_3$.

| Manometer | Reaction system | Primary reaction products | Final products of secondary reaction | Final $O_2$ uptake ($\mu$l.) |
|---|---|---|---|---|
| 1 | Oxidase + alanine + $O_2$ | $CH_3.CO.COOH + NH_3 + H_2O_2$ | $CH_3.COOH + NH_3 + CO_2$ | 340 |
| 2 | Oxidase + alanine + catalase + $O_2$ | | $CH_3.CO.COOH + NH_3 + \frac{1}{2}O_2$ | 190 |
| 3 | Oxidase + alanine + ethanol + $O_2$ | | $CH_3.COOH + NH_3 + CO_2$ | 362 |
| 4 | Oxidase + alanine + ethanol + catalase + $O_2$ | | $CH_3.CO.COOH + NH_3 + CH_3CHO$ | 350 |

from catalase, and with this preparation we carried out a series of manometric experiments.

In the right-hand flasks of four Barcroft manometers were placed 0·5 ml. of enzyme fraction *D* and excess (50 $\mu$g.) of flavine adenine dinucleotide, while 0·3 ml. 1% *dl*-alanine (equivalent to 188 $\mu$l. $O_2$) was put in a dangling tube in each flask. Manometer 2 received 0·2 ml. of strong catalase solution, manometer 3 received 0·1 ml. 10% ethanol, while the fourth manometer received both ethanol and catalase. 0·3 ml. 10% KOH and a roll of filter paper were placed in the central tubes to absorb $CO_2$. The volumes were made up to 3 ml. with 0·07 M-pyrophosphate pH 8·3.

The results of these experiments, summarized in Table 1, show that the addition of catalase to the primary oxidation system halved its $O_2$ uptake owing to the decomposition of $H_2O_2$ and liberation of $O_2$. Catalase thus protects the pyruvate from oxidation and brings the $O_2$ uptake down to the theoretical amount. When, however, both catalase and ethanol are added to the primary oxidation system the $O_2$ uptake again becomes double. In this case ethanol and not pyruvate is oxidized by the $H_2O_2$, the latter being preferentially utilized by catalase for catalysis of the coupled oxidation of ethanol.

It may be mentioned here that, according to Sevag & Maiweg (1934), pyruvate as well as catalase will protect pneumococci from the toxic effect of the $H_2O_2$ formed when these micro-organisms are exposed to air.

*Coupled oxidation of other alcohols*

Although the oxidation of other alcohols was investigated with each of the three primary oxidizing systems, we shall refer now only to results obtained with *d*-amino-acid oxidase and glucose oxidase. These experiments were carried out in the same manner as those with xanthine oxidase.

In the case of crude amino-acid oxidase the right-hand flasks of each manometer received a phosphate buffer extract of 50 mg. of pig's kidney powder and catalase, while 0·3 ml. 1% *dl*-alanine was put into the dangling tube. With the exception of the control the flasks received 5–20 mg. of different alcohols. In the case of glucose oxidase, the right-hand flasks received 50 $\mu$g. glucose oxidase and the dangling tube contained 0·3 ml. 1% glucose, the other ingredients being added as before.

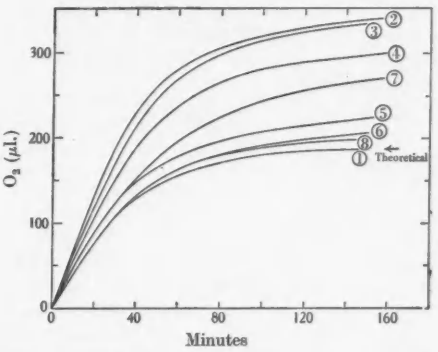


Fig. 3. Coupled oxidation of alcohols by crude *d*-amino-acid oxidase system (= extract of 50 mg. kidney powder + 3 mg. *dl*-alanine). Temp. 39°; pH 7·3. In addition to oxidase system + catalase the following substances were present: (1) none (control); (2) 5 mg. methanol; (3) 5 mg. ethanol; (4) 10 mg. *n*-propanol; (5) 10 mg. *iso*propanol; (6) 15 mg. *n*-butanol; (7) 15 mg. *iso*butanol; (8) 20 mg. amyl alcohol.

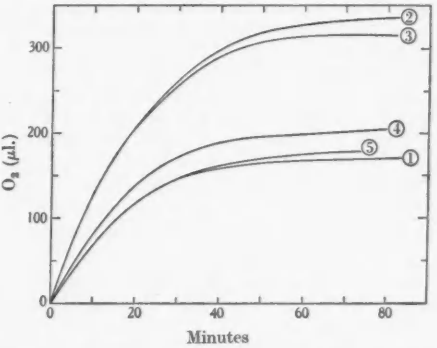


Fig. 4. Coupled oxidation of alcohols by the glucose oxidase (notatin) system: 50 $\mu$g. notatin + 0·3 ml. 1% glucose. Temp. 39°; pH 6·0. In addition to oxidase system + catalase the following substances were present: (1) none (control); (2) 5 mg. methanol; (3) 5 mg. ethanol; (4) 10 mg. *n*-propanol; (5) 10 mg. *iso*propanol.

Higher alcohols were not oxidized.

The results of these experiments, given in Figs. 3 and 4, show that the alcohols which most readily undergo coupled oxidation in presence of catalase and a primary oxidation system are methanol and ethanol whereas *n*-propanol and *iso*butanol are much less readily attacked. Similar results have been obtained with uricase (Keilin & Hartree, (1936). Ethylene glycol and β-amino-ethanol are oxidized as readily as ethanol (Fig. 5), but since

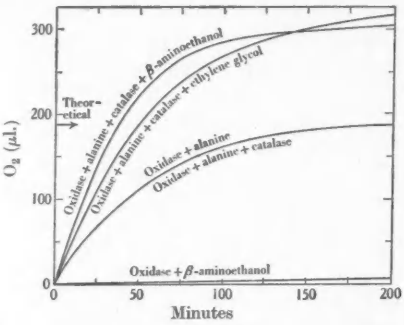


Fig. 5. Coupled oxidation of β-amino-ethanol and ethylene glycol by the *d*-amino-acid oxidase system. Experimental conditions as for Fig. 3. 5 mg. ethylene glycol, 5 mg. β-amino-ethanol.

glycollic aldehyde is not oxidized, this substance appears to be the oxidation product of ethylene glycol. Glycerol and choline, however, are not oxidized. In Table 2 are listed the substances which have been tested and found not to undergo coupled oxidation.

Table 2. *Substances which do not undergo coupled oxidation in presence of catalase*

| | | |
|---|---|---|
| *n*-Octanol | Dulcitol | Glycollic aldehyde |
| Benzyl alcohol | Pentaerythritol | Glycollic acid |
| Choline | Xylose | Dihydroxymaleic acid |
| Glycerol | Glucose | Lactic acid |
| Erythritol | Fructose | Malic acid |
| Mannitol | Galactose | Tartaric acid |
| Inositol | | |

*Relative concentrations of catalase required for the decomposition of $H_2O_2$ and for its utilization in the coupled oxidation of alcohol*

We have shown above that catalase added to a primary oxidation system can catalyze two distinct reactions: (1) ordinary catalatic decomposition of $H_2O_2$ formed in the primary reaction, and (2) utilization of this peroxide for a secondary oxidation of alcohols. In a primary oxidation system like glucose oxidase, the former reaction halves the total $O_2$ uptake, bringing it down to the theoretical level for the oxidation of the substrate, whereas in the second reaction the $O_2$ uptake is again double the theoretical value (Table 3).

It was of interest to determine the minimum concentrations of catalase which are required for promoting these reactions (3 and 4 in Table 3). For this purpose a series of manometric experiments was carried out, using glucose oxidase as the primary oxidation system with and without ethanol and with different concentrations of purified catalase.

The results of these experiments, summarized in Table 4, clearly show that the concentration of catalase required for oxidation of alcohols by means of the $H_2O_2$ formed in the primary oxidation is about 1000 times greater than that required for its catalatic decomposition into $O_2$ and water.

Table 4. *Effect of varying concentrations of catalase on the system glucose oxidase (enzyme) + glucose ± ethanol*

(Theoretical $O_2$ uptake (3 mg. glucose) = 187 μl. Catalase concentration expressed in terms of haematin.)

| Contents of flasks | Total $O_2$ uptake (μl.) |
|---|---|
| Enzyme + glucose ± ethanol | 345 |
| Enzyme + glucose + catalase ($2 \times 10^{-10}$ M) | 250 |
| Enzyme + glucose + catalase ($2 \times 10^{-9}$ M) | 187 |
| Enzyme + glucose + ethanol + catalase ($2 \times 10^{-8}$ M) | 240 |
| Enzyme + glucose + ethanol + catalase ($2 \times 10^{-7}$ M) | 302 |
| Enzyme + glucose + ethanol + catalase ($2 \times 10^{-6}$ M) | 348 |

*Nature of the peroxide required for oxidation of ethanol by catalase*

We have shown previously that ethanol could be oxidized by $H_2O_2$ deriving either from a primary oxidation reaction or from decomposition of barium and cerium peroxides, but not by $H_2O_2$ added as a solution (Keilin & Hartree, 1936). It appeared therefore that the peroxide acting in this reaction was in a specially active state which we have described as a 'nascent state'. It is, however, possible that it is not the supposed higher level of activity of the 'nascent peroxide' which enables catalase to

Table 3. *Analysis of the effect of ethanol and catalase on the system glucose oxidase + glucose*

| | Reaction system | Primary reaction products | Secondary reaction induced by catalase | Net $O_2$ uptake* |
|---|---|---|---|---|
| 1 | Oxidase + glucose + $O_2$ | Gluconic acid + $H_2O_2$ | None | 2*n* |
| 2 | Oxidase + glucose + ethanol + $O_2$ | | None | 2*n* |
| 3 | Oxidase + glucose + catalase + $O_2$ | | $H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$ | *n* |
| 4 | Oxidase + glucose + catalase + ethanol + $O_2$ | | $H_2O_2 + C_2H_5OH \rightarrow CH_3CHO + 2H_2O$ | 2*n* |

* *n* = theoretical $O_2$ uptake for oxidation of glucose to gluconic acid: $C_6H_{12}O_6 + \frac{1}{2}O_2 = C_6H_{12}O_7$.

utilize it for the oxidation of ethanol, but rather the presence of the peroxide in a low concentration throughout the reaction. If this is the case ethanol should undergo oxidation in presence of catalase by free $H_2O_2$ provided the latter is supplied slowly and continuously. The slow delivery of $H_2O_2$ into a mixture of catalase and ethanol can be assured by at least three different methods: (1) volatilization at 39° of a solution of $H_2O_2$, (2) addition of $H_2O_2$ drop by drop combined with efficient stirring, and (3) addition of $H_2O_2$ in the form of a fine mist produced by a suitable atomizer.

(1) Experiments with the addition of $H_2O_2$ by volatilization were carried out in manometer flasks. For this purpose three differential manometers were used and their right-hand flasks were filled as follows: (i) 0·3 ml. catalase; (ii) 0·3 ml. catalase + 0·1 ml. 20% ethanol; (iii) 0·1 ml. 20% ethanol. The volumes in the flasks were made up to 3 ml. with 0·1 M-phosphate buffer pH 6·0 and 0·3 ml. 30% hydrogen peroxide (perhydrol) was put into the central tube of each flask. The manometers were shaken for 7 hr. in a bath at 39° with the taps open. In these experiments peroxide gradually diffuses as vapour into the solution in the flasks. This $H_2O_2$ may be partly decomposed catalatically and partly used for oxidation of ethanol. The latter reaction is very slow except in presence of catalase as is shown by estimations of acetaldehyde (Table 5, *a*).

Table 5. *The catalytic oxidation of ethanol by gradual addition of* $H_2O_2$ *in presence of catalase:*

(*a*) Diffusion of $H_2O_2$ vapour. (*b*) Dropwise addition of $H_2O_2$. (*c*) Addition of $H_2O_2$ as mist.

| | Contents of flasks | Acetaldehyde formed (μg.) after slow addition of $H_2O_2$ | | |
|---|---|---|---|---|
| (*a*) | Catalase + ethanol | 200 | 400 | 100 |
| | Catalase | 10 | 15 | 0 |
| | Ethanol | 40 | 50 | 5 |
| (*b*) | Catalase + ethanol | 1050 | | |
| | Ethanol | 175 | | |
| (*c*) | Catalase + ethanol | 2500 | | |
| | Ethanol | 80 | | |

(2) Experiments with a continuous and slow addition of a solution of $H_2O_2$ were carried out at room temperature. For this purpose 20 ml. 0·7 N-$H_2O_2$ were added slowly during 35 min. to a mixture of 40 ml. 0·1 M-phosphate buffer pH 6·0, 10 ml. 10% ethanol and 1 ml. catalase (≡ 0·18 mg. haematin) in a 150 ml. flask. The $H_2O_2$ was introduced drop by drop while the mixture was stirred very rapidly. In a control experiment without catalase 1 drop of the latter was added at the end of the experiment in order to decompose the accumulated $H_2O_2$. At the completion of the experiments the aldehyde was estimated and the results are shown in Table 5, *b*.

(3) Experiments with the addition of $H_2O_2$ solution in the form of a mist were carried out as follows: $N_2$ from a cylinder, after being heated to 40°, was passed via active charcoal to an atomizer containing 2·4 g. perhydrol. After trapping the larger droplets, the mist of $H_2O_2$ was passed into a 500 ml. flask containing 5 ml. 0·1 M-phosphate buffer pH 6·0, 5 ml. 5% ethanol and 1 ml. catalase (≡ 0·18 mg. haematin). The issuing gas escaped via a liquid air trap. The apparatus was set up in a shaker and the experiment was run for 1 hr. A control experiment was carried out in absence of catalase. At the end of the experiment the excess of $H_2O_2$ in the control was decomposed by the addition of 1 drop of catalase solution and the contents of the flask plus liquid air trap were analyzed in both cases for acetaldehyde (Table 5, *c*).

All these experiments clearly show that in presence of catalase ethanol can be oxidized to aldehyde not only by 'nascent peroxide' but also by a solution of free $H_2O_2$. There is, however, a very marked difference in the efficiency of these two processes. Whereas in the coupled oxidation of ethanol almost the entire amount of $H_2O_2$ formed in the primary reaction is used for the secondary oxidation process, thus oxidizing an equivalent amount of ethanol, in the oxidation of ethanol by a solution of $H_2O_2$ and catalase only a small fraction of $H_2O_2$ is utilized for this purpose, the main portion being decomposed catalatically. It does, therefore, appear to be impossible to devise an efficient method of continuous delivery of free $H_2O_2$, the concentration of which throughout the solution and during the whole course of the experiment could be maintained at the optimum low value.

### *The possible functions of catalase*

It is generally believed that catalase by catalyzing the decomposition of $H_2O_2$ protects cells and tissues from its toxic effect. That catalase is capable of protecting an enzyme such as xanthine oxidase from destruction by $H_2O_2$ formed during the catalytic oxidation of hypoxanthine was well demonstrated by Dixon (1925) and confirmed by other workers (see also Fig. 2). In this connexion it is interesting to note that liver and kidney, which are rich in catalase, are also rich in enzyme systems such as uricase, amino-acid oxidase, tyraminase and others which, *in vitro*, are capable of reacting directly with $O_2$, reducing it to $H_2O_2$. On the other hand, the non-nucleated erythrocytes of mammals, which also contain a very high concentration of catalase, have hardly any respiratory or oxidative activity, whereas nucleated erythrocytes of certain birds which show a marked respiratory activity contain very little catalase.

It has been suggested by Bingold (1933) that the function of catalase within corpuscles is the protection of haemoglobin against oxidative destruction by $H_2O_2$ formed in different tissues, especially in the kidney. That endoerythrocytic catalase, under certain experimental conditions, is capable of protecting haemoglobin from oxidation by $H_2O_2$ to methaemoglobin can easily be demonstrated. Thus, on treating mammalian red blood corpuscles with a solution of $H_2O_2$, the latter undergoes a

violent decomposition accompanied by the liberation of $O_2$; the oxyhaemoglobin, however, remains almost completely unchanged. Only when the endoerythrocytic catalase is inhibited by cyanide, azide or hydroxylamine can the oxyhaemoglobin be oxidized to methaemoglobin by $H_2O_2$. On treating bird erythrocytes with $H_2O_2$ the latter undergoes a very slow catalatic decomposition while oxyhaemoglobin is rapidly oxidized.

At first sight these experiments seem to support Bingold's view that endoerythrocytic catalase protects oxyhaemoglobin from oxidation by $H_2O_2$. However, further study of this problem revealed several points at variance with this view. First of all there is a marked difference between the experimental conditions described above and the conditions within a living organism. Thus, in our experiments *in vitro* $H_2O_2$ was used in fairly high concentrations, whereas in cells or tissues of living organisms $H_2O_2$, if it is formed, must be present in a very low concentration. In fact, the $H_2O_2$ may derive only from oxidizing systems which, like xanthine oxidase, can react directly with $O_2$, reducing it to $H_2O_2$. Since the affinities of these systems for $O_2$ are distinctly smaller than that of the cytochrome system, and as the tension of $O_2$ within cells and tissues is usually very low, little peroxide may be expected to derive from this source. The best way of testing the protective function of endoerythrocytic catalase under conditions approaching those *in vivo* is to bring intact mammalian erythrocytes in contact with a primary oxidation system reducing $O_2$ to $H_2O_2$.

For this purpose 40$\mu$g. glucose oxidase and 0·3 ml. 6% glucose are mixed with 2·7 ml. isotonic phosphate buffer pH 7·3 and 0·3 ml. washed horse erythrocytes. The mixture is shaken at 39°. After 5 min. shaking about 75%, and after 15 min. 100% of the haemoglobin is found to be oxidized to methaemoglobin. Experiments carried out with laked corpuscles gave similar results, the amount of oxyhaemoglobin converted to methaemoglobin after 5, 15 and 25 min. shaking being approximately 50, 90 and 100% respectively. The oxidation of haemoglobin was not affected by the addition of ethanol, which simultaneously underwent coupled oxidation to aldehyde. Similar experiments were carried out with oxyhaemoglobin which had been recrystallized several times until free from catalase. In this case not only was oxyhaemoglobin oxidized to methaemoglobin, but a portion of the pigment underwent an irreversible oxidative destruction of its tetrapyrrolic ring accompanied by denaturaton of the protein and the formation of green verdohaemochromogen.

These experiments show that endoerythrocytic catalase does not protect haemoglobin from oxidation to methaemoglobin by very small concentrations of $H_2O_2$ formed in a primary oxidation reaction. In other words, under natural conditions the $H_2O_2$, if formed in such reactions, should be capable of oxidizing the divalent iron of haemoglobin in spite of the large concentration of endoerythrocytic catalase. If such an oxidation does not take place it is certainly not due to the protective action of catalase but rather to the fact that $H_2O_2$ does not come in contact with the haemoglobin.

We may now ask how catalase can protect haemoglobin from oxidation by a great excess of $H_2O_2$ if it does not protect it against a very small concentration of $H_2O_2$ such as may be formed *in vivo*. On addition of a large amount of $H_2O_2$ to a suspension of red blood corpuscles the $H_2O_2$ is violently decomposed into $O_2$ and water. The solution becomes saturated with $O_2$ and the equilibrium reaction $HbO_2 \rightleftharpoons Hb + O_2$ is shifted towards the left, thus reducing the concentration of Hb. Since it is the unoxygenated haemoglobin (Hb) that reacts with $H_2O_2$ and thus undergoes oxidation, the removal of it will reduce the rate of formation of methaemoglobin. This is analogous to the autoxidation of haemoglobin to methaemoglobin by $O_2$. In fact, it was shown by Neill & Hastings (1925) and by Brooks (1929, 1935) that the optimum rate of oxidation of oxyhaemoglobin by $O_2$ is at small tensions of the gas. According to Brooks (1935) the rate of autoxidation of Hb at different $O_2$ pressures is proportional to the concentration of Hb and to a function of the $O_2$ pressure. In this reaction $O_2$ has a triple function: (1) the determination of the concentration of Hb, (2) the oxidation of Hb, and (3) the inhibition of this oxidation by forming oxyhaemoglobin.

If we assume that $H_2O_2$ is formed in cells as a result of certain oxidation reactions, it is unlikely that cells are protected from its toxic action only by catalatic decomposition of this $H_2O_2$. In fact we have already seen that endoerythrocytic catalase is unable to protect haemoglobin from oxidation by small amounts of $H_2O_2$, yet neither haemoglobin nor myoglobin are oxidized within the living organism. Moreover, the formation of $H_2O_2$ and its catalatic decomposition would be a very uneconomic process. It is more likely that the $H_2O_2$, if formed, is utilized in coupled oxidations. That the main function of catalase may be the catalysis of such oxidative reactions by means of $H_2O_2$ is supported by another consideration. We have seen that the concentration of catalase required for the oxidation of ethanol by means of $H_2O_2$ formed in a primary oxidation reaction is about $2 \times 10^{-6}$ M (estimated in terms of haematin) which is about 1000 times greater than the concentration ($2 \times 10^{-9}$ M) required for the catalatic decomposition of this $H_2O_2$. The concentration of catalase in liver and in non-nucleated erythrocytes of mammals, which is of the order of $4 \times 10^{-6}$ M, would be adequate for oxidative utilization of the $H_2O_2$ produced by these tissues on the assumption that all the $O_2$ utilized during respiration is reduced to $H_2O_2$. At present coupled oxida-

tion may be considered as a much more likely biological function of catalase than the decomposition of $H_2O_2$ into $O_2$ and water. This, however, does not exclude the possibility that catalase may have yet another function.

## DISCUSSION

Oxidizing enzymes such as xanthine oxidase, uricase, *d*-amino-acid oxidase, tyraminase, glucose oxidase and others, which catalyze the oxidation of their respective substrates by molecular $O_2$, reduce the latter to $H_2O_2$. The total $O_2$ uptake by such systems should therefore be double the amount calculated for the oxidation of the substrate. This can be demonstrated experimentally provided that (*a*) the reaction is carried out in the complete absence of catalase, (*b*) the peroxide formed in the reaction does not inactivate the enzyme, and (*c*) the peroxide formed in the oxidation of one molecule of the substrate does not act as hydrogen acceptor in the oxidation of a further molecule of the same substrate. However, on addition of a small amount of catalase to these systems, the velocities of their reactions are reduced and the total $O_2$ uptake is brought down to the theoretical amount, since $H_2O_2$ formed in the primary reaction is decomposed catalatically, liberating $O_2$.*

Addition of ethanol to primary oxidizing systems already containing catalase doubles the total $O_2$ uptake by these systems since $H_2O_2$ formed in the primary reaction is utilized for the secondary or coupled oxidation of ethanol. The oxidation of ethanol by $H_2O_2$ takes place only in presence of catalase which catalyzes this reaction instead of abolishing it by splitting $H_2O_2$ into $O_2$ and water. The fact that the coupled oxidation of ethanol is catalyzed by a highly purified or crystalline catalase shows that no other enzyme is involved in this reaction.

In a system composed of the Schardinger enzyme, acetaldehyde, catalase and ethanol, $H_2O_2$ formed in the primary oxidation of aldehyde to acid is utilized by catalase for the oxidation of ethanol to acetaldehyde. Thus the product of the secondary oxidation supplies the substrate for the primary oxidation system. This 'cyclic oxidation' reaction, which proceeds until all ethanol is used up, can also be catalyzed by highly purified catalase.

In addition to ethanol, purified catalase promotes the oxidation of other alcohols such as methanol, ethylene glycol, β-aminoethanol, and to a lesser degree *n*-propanol and *iso*butanol.

* This point must be borne in mind in calculating the activities of enzymes during purification. In fact, the apparent increase in the activity of uricase (Holmberg, 1939) and of amino-acid oxidase (Negelein & Brömel, 1939) during their purification is partly due to the removal of catalase.

For the catalytic oxidation of ethanol by catalase, $H_2O_2$ formed in a primary oxidation reaction can be replaced by barium or cerium peroxide or even by free $H_2O_2$ provided that the latter is added so slowly and continuously that its concentration remains very low. However, the primary oxidation reaction is by far the most efficient mechanism of supply of $H_2O_2$ since nearly every molecule of $H_2O_2$ thus formed is utilized in the oxidation of the ethanol. In all other methods of addition of $H_2O_2$ only a small fraction of added $H_2O_2$ is used in this reaction, the main portion being decomposed catalatically. Such methods show nevertheless that the high efficiency of $H_2O_2$ formed in the primary oxidation reaction is not due to a 'nascent' or active form of this peroxide but to a more suitable manner of its supply to the catalase-ethanol system.

The concentration of catalase which is required for catalatic decomposition of $H_2O_2$ formed in a primary oxidation reaction is about 1000 times lower than that which is required for the coupled oxidation of ethanol. This explains why some crude enzyme preparations containing traces of catalase give with their substrates theoretical $O_2$ uptakes and yet are unable to catalyze the coupled oxidation of ethanol.

As to the function of catalase, it is far from being elucidated in spite of the facts that it is one of the most widely distributed intracellular enzymes, that it can easily be obtained in a pure crystalline state, and that its structure as well as the kinetics of the catalytic decomposition of $H_2O_2$ are well determined. In this respect the position of catalase resembles that of peroxidase and phenol oxidases which can also be obtained in pure states. Although the structure and the intimate mechanism of the reactions they catalyze are well understood, very little is known as to the part they play in cellular metabolism.

Since the work of Thénard (1811), Schönbein (1863 *a*, *b*) and Loew (1901) it has been considered that the main if not the only function of catalase is protective and consists in the decomposition of $H_2O_2$ into $O_2$ and water ($2H_2O_2 = 2H_2O + O_2$). The fact that this reaction is violent and is readily detected and measured directly with cells and tissues has tended to rule out any inquiries into other possible functions of the enzyme.

This idea of a protective function of catalase tacitly assumes that $H_2O_2$ is formed *in vivo* as a result of intracellular oxidation reactions. That a small amount of $H_2O_2$ may be formed within cells is supported by the following few considerations:

(*a*) The formation of $H_2O_2$ has been demonstrated *in vitro* as a result of oxidation reactions catalyzed by several intracellular oxidizing enzymes which react directly with $O_2$. In certain of these cases it has even been shown that catalase protects the enzymes against the toxic effect of $H_2O_2$.

(*b*) Catalase and peroxidase, which are the only enzymes reacting *in vitro* with $H_2O_2$, to some extent exclude each other. Thus catalase, although universally distributed, reaches the highest concentration in animals, whereas peroxidase is widely distributed in plants, and in animals is known only in milk and in leucocytes.

(*c*) $H_2O_2$ can easily be detected in culture media of certain bacteria, devoid of catalase, when they are exposed to air, as is the case with pneumococci (see p. 295) and with lactic acid bacteria (*B. delbrückii*, *acidophilus* or *bulgaricus*). It has also been found in culture media of certain moulds such as *Penicillium* and *Aspergillus*.

Contrary to the view that $H_2O_2$ is generally formed in cells and tissues during respiratory processes are the following two facts: (1) although $H_2O_2$ can be detected in certain isolated enzyme systems, it has not, with very few exceptions, been detected in highly respiring cells of strictly aerobic organisms even when the catalase is inhibited by hydroxylamine, and (2) enzyme systems which *in vitro* reduce $O_2$ to $H_2O_2$ usually have small affinities for $O_2$, and as the tension of $O_2$ within tissues of higher organisms is very low it is conceivable that these systems do not react directly with $O_2$ but through other systems which do not give rise to $H_2O_2$.

If, however, we accept the idea that $H_2O_2$ is formed within cells of aerobic organisms, it is unlikely that the main function of catalase would consist in splitting this peroxide into $O_2$ and water in order to protect cells and their enzymes from its toxic effect, as was shown for the xanthine system *in vitro* (Dixon, 1925). That the function of catalase is not merely a protective one is supported by the following considerations:

(*a*) A high concentration of catalase in mammalian red blood corpuscles does not protect haemoglobin against oxidation to methaemoglobin by $H_2O_2$ formed in a primary oxidation reaction.

(*b*) The presence of catalase in *Bacillus subtilis*, *Proteus vulgaris* and *Escherichia coli* does not protect these micro-organisms against the toxic effect of very small concentrations of $H_2O_2$ (1 in 4,000,000) added directly to the media or formed as the result of the autoxidation of ascorbic acid (Lwoff & Morel, 1942). Neither does the presence of catalase in *Staphylococcus aureus* afford protection against very low concentrations of $H_2O_2$ formed during the oxidation of glucose (Coulthard *et al.* 1942; Birkinshaw & Raistrick, 1943) or of hypoxanthine (Lipmann & Owen, 1943) catalyzed by their respective enzymes.

(*c*) If we assume that the rate of $H_2O_2$ formation in cells of liver is equivalent to the rate of $O_2$ uptake, the concentration of catalase in these cells is at least 1000 times higher than is necessary for an efficient catalatic decomposition of this peroxide but is of the right order for catalysis of coupled oxidation.

(*d*) Catalatic decomposition of $H_2O_2$, liberating $O_2$ for further oxidation reactions, is a wasteful process which can hardly be considered as a general mode of utilization of $H_2O_2$ formed in some primary oxidations.

The catalysis of coupled oxidation by catalase appears therefore to be a more likely biological property of this enzyme than the decomposition of $H_2O_2$ into $O_2$ and water. It is conceivable that in addition to alcohols catalase may promote a peroxidatic oxidation of other biologically important substances. On the other hand, the fact that catalase is present in very high concentrations in the non-nucleated and hardly respiring erythrocytes of mammals suggests that it may have yet another physiological function not necessarily connected with $H_2O_2$.

## SUMMARY

1. Certain oxidizing enzymes, in catalyzing the oxidation of their substrates by molecular $O_2$, reduce the latter to $H_2O_2$, which in presence of catalase is decomposed as follows:

$$2H_2O_2 = O_2 + 2H_2O. \quad (1)$$

Pure catalase may alternatively utilize the $H_2O_2$ formed in such primary oxidation reactions for catalysis of the secondary or coupled oxidation of alcohols to aldehydes:

$$R{\cdot}CH_2OH + H_2O_2 = R{\cdot}CHO + 2H_2O. \quad (2)$$

2. For catalysis of this coupled oxidation (2), $H_2O_2$ formed in the primary oxidation can be replaced by $H_2O_2$ resulting from the slow decomposition of barium or cerium peroxides, or by free $H_2O_2$ provided the concentration of the latter is kept low by very gradual addition. However, $H_2O_2$ deriving from a primary oxidation is much more efficient in this reaction, since every molecule of $H_2O_2$ thus formed can be used in the coupled oxidation of alcohols, whereas in all other forms of addition of $H_2O_2$ reaction (1) greatly predominates.

3. In presence of xanthine oxidase and aldehyde, purified catalase also promotes a cyclic oxidation of ethanol to acetic acid, which proceeds until all alcohol is oxidized.

4. In a system composed of purified *d*-amino-acid oxidase and alanine, the $H_2O_2$ formed is utilized in a non-catalyzed coupled oxidation of pyruvate to acetate and $CO_2$. If, however, catalase and ethanol are added to this system, $H_2O_2$ is utilized for the coupled oxidation of the ethanol, leaving the pyruvate unattacked.

5. The high concentration of endoerythrocytic catalase does not protect the divalent iron of haemoglobin from oxidation by $H_2O_2$ formed in a primary

oxidation system. Similarly the presence of catalase in micro-organisms does not protect them from the toxic effect of $H_2O_2$ added as such or formed in the medium as a result of certain oxidation reactions. It is therefore unlikely that the main function of catalase consists in the decomposition of $H_2O_2$ into $O_2$ and water in order to protect cells from its toxic effect.

6. The catalysis of coupled oxidation by means of $H_2O_2$ formed in a primary oxidation is a more likely biological property of catalase. In fact, the concentration of catalase in liver tissues appears to be about 1000 times higher than that required for the catalatic decomposition of $H_2O_2$, and is of the same order as is found to be necessary for catalysis of coupled oxidation.

7. So far only methanol, ethanol, *n*-propanol, *iso*butanol, β-amino-ethanol and ethylene glycol have been shown to undergo coupled oxidation catalyzed by catalase *in vitro*. It is possible that *in vivo* other biologically important compounds may undergo similar oxidations.

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# The Urinary Partition of Sulphur in Rats Treated with Aromatic Hydrocarbons, with Special Reference to Growth Retardation


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(*Received* 30 *May* 1945)


Haddow, Scott & Scott (1937) found that certain carcinogenic hydrocarbons produced immediate, constant, and long-continued reduction in the growth rate of young rats, while some non-carcinogenic hydrocarbons did not show this effect. White & White (1939) produced growth retardation in rats by the incorporation of methylcholanthrene, 3:4-benzpyrene, or pyrene in the diet. Later the same workers (1940) observed no difference between the growth effects produced by single intraperitoneal injections of carcinogenic or non-carcinogenic hydrocarbons; both types retarded growth.

White & White concluded, on the basis of their own results and from a consideration of data available in the literature for bromobenzene, naphthalene, and anthracene, that the growth retardation produced by the hydrocarbons is due to a specific deficiency of the sulphur-containing amino-acids imposed on the animals by an increased requirement for organic sulphur, in the form of cystine or methionine, for detoxication mechanisms that involve the formation of mercapturic acids. They emphasized the desirability of actual isolation of the postulated mercapturic acids in order to demonstrate conclusively the relation between growth effects and sulphur metabolism.

Stekol (1943) showed that the addition of bromobenzene to the diet of rats produces, in effect, a decrease in available protein proportional to the mercapturic acid excreted. Growth retardation does not occur on a high protein diet but increases as the protein content is progressively lowered. Bromobenzene, composition of diet, and the degree of growth retardation are quantitatively related.

The mechanism of the growth retardation produced by these hydrocarbons is important, since

some of them are capable of retarding the growth of neoplasms (Haddow, 1935; Haddow & Robinson, 1937). It appeared probable that some light might be thrown on the possible significance of sulphur metabolism in this connexion by a quantitative examination of the sulphur partition in the urine of rats treated with selected aromatic hydrocarbons. In particular, the formation of mercapturic acids should be apparent from estimations of the neutral sulphur fraction. The experiments described in the present paper were designed to investigate this point. At the same time they afforded a comparison of the relative growth-retarding effects of aromatic hydrocarbons of differing structures and complexity.

## MATERIALS AND METHODS

*Animals.* All experiments were carried out on male rats. During the experimental period the animals were maintained on a standard diet of the following composition: national wheatmeal flour (85% extraction) 72%, casein 11%, milk powder (full cream) 8%, Bemax (Vitamins Ltd.) 2·5%, margarine 3%, $CaCO_3$ 1%, salt mixture (Glaxo Laboratories Ltd.) 1%, cod-liver oil 1·5%. This diet contains approximately 25% of protein and is rich in sulphur (230 mg./100 g.). Rats grow well on it, the daily weight increases for male rats being 3–4 g. Groups of ten rats were kept in metabolism cages and supplied with this diet and water *ad libitum*. Each group was weighed daily throughout the experiments. At the beginning of the control period each rat received 1 ml. of arachis oil by intraperitoneal injection. During the week following this control injection four estimations of the daily excretion of inorganic sulphate, ethereal sulphate, and neutral sulphur were made. At the end of the control period the hydrocarbon was administered in arachis oil by the intraperitoneal route. The volume of oil was always 1 ml., and this contained the appropriate amount of hydrocarbon in solution or as a fine suspension. Daily estimations of urinary sulphur partition were continued for varying periods after this injection. In addition to the sulphur estimations a determination of glucuronic acid was also carried out on each specimen of urine by a method based on that described by Maughan, Evelyn & Browne (1938).

*Sulphur determinations.* These were carried out by the procedure described for human urines (Peters & Van Slyke, 1932). At first the attempts to apply this volumetric benzidine method to rat urines gave disappointing results, although it worked well with human urine. It was found that the introduction of small but important changes in the usual procedure led to a method that gave reproducible results. Careful attention to the following points was found to be necessary.

(*a*) The use of a more dilute aqueous acetone (75 ml. acetone diluted to 100 ml.) for washing the precipitated benzidine sulphate rather than the 80% or higher concentrations usually recommended was arrived at after many trials. It was found that the higher concentrations of acetone tended to precipitate benzidine hydrochloride on the filter and led to values for sulphur that were too high. Acetone in approximately 75% (v/v) concentration is safe in this respect and may be freely used for washing, since it does not dissolve any appreciable amount of precipitate.

(*b*) The rat urine was diluted tenfold and 5 ml. portions used for estimations. If larger volumes (or higher concentrations) are used, the end-point of the total sulphate estimation gives difficulty owing to the presence of brown colouring matter. The use of small volumes has the additional advantage that, provided a brisk ebullition is maintained, the precipitate dissolves during the course of the titration and it is unnecessary to break up the benzidine sulphate mechanically.

(*c*) Whatman no. 50 filter papers are more satisfactory in use than paper-pulp filters.

(*d*) In determining total sulphur it is essential to use a large excess of Benedict's sulphur reagent. The proportion of neutral sulphur is higher in rat than in human urine.

As an example of the use of the method the results of a study of normal male rats are shown in Table 1.

The hydrocarbons investigated were: benzene, naphthalene, phenanthrene, anthracene, 1:2-benzanthracene, 1:2:5:6-dibenzanthracene, 3:4-benzpyrene, pyrene, and chrysene. Preliminary purification through the picrate or by chromatographic adsorption was carried out where necessary.

## RESULTS

The daily excretions/100 g. rat of inorganic sulphate, ethereal sulphate, and neutral sulphur are shown in Figs. 1–3, both during a preliminary control period and after injection of the hydrocarbons. In Fig. 4 the effects of the various hydrocarbons on the growth

Table 1. *The daily excretion of inorganic sulphate, ethereal sulphate and neutral sulphur in the urine of normal male rats*

(Data for urinary sulphur are expressed as mg. of sulphur/day. Highest and lowest figures are given in parentheses.)

| No. of determinations | Body wt. (g.) | Inorganic sulphate | | Ethereal sulphate | | Neutral sulphur | |
|---|---|---|---|---|---|---|---|
| | | per rat | per 100 g. rat | per rat | per 100 g. rat | per rat | per 100 g. rat |
| 30 | 80–100 | 2·7 (1·2–3·9) | 3·0 (1·4–4·4) | 0·21 (0–0·40) | 0·24 (0–0·50) | 0·9 (0·4–1·8) | 1·0 (0·5–1·9) |
| 18 | 100–120 | 2·9 (1·8–3·9) | 2·6 (1·6–3·6) | 0·22 (0–0·36) | 0·20 (0–0·35) | 1·6 (0·8–2·6) | 1·4 (0·6–2·2) |
| 20 | 120–140 | 3·3 (1·7–4·7) | 2·6 (1·4–3·5) | 0·23 (0–0·40) | 0·18 (0–0·30) | 1·4 (0·6–2·6) | 1·1 (0·5–2·1) |

rate of the rats are illustrated. Increased excretions of ethereal sulphate, neutral sulphur, and glucuronic acid, together with the corresponding growth rates, are shown in Table 2.

Table 2. *The effect of intraperitoneal injections of aromatic hydrocarbons on the excretion of urinary sulphur and glucuronic acid and on the rate of growth of male rats*

(Only increased excretions are indicated for sulphur and glucuronic acid. Growth rates marked * are the steady rates reached after a short period of weight loss.)

| Hydrocarbon | Ethereal sulphate | Neutral sulphur | Glucuronic acid | Growth rate (g./day) Control period | Growth rate (g./day) After injection |
|---|---|---|---|---|---|
| Benzene | + + + | – | – | 4·3 | 4·3 |
| Naphthalene (46 mg.) | + + | + + + + | + + + + | 3·9 | 4·9 |
| Naphthalene (86 mg.) | + | + + + + + + | + + + + + + | 3·0 | 3·0* |
| Phenanthrene | + + + | + + | + + | 4·4 | 2·6* |
| Anthracene | – | + | + + + | 4·7 | 2·8 |
| 1:2-Benzanthracene | – | + | + | 3·5 | 1·6 |
| 1:2:5:6-Dibenzanthracene | – | + – | · | 3·3 | 0·0 |
| 3:4-Benzpyrene | – | – | + | 3·6 | 0·0 |
| Pyrene | + + + | + + | + + | 4·3 | 3·1* |
| Chrysene | – | – | – | 4·1 | 2·0* |

Results with the individual hydrocarbons are summarized under the name of the hydrocarbon in question.

*Benzene.* Rats receiving 86 mg./100 g. body weight showed a decreased excretion of inorganic sulphate with concomitant rise in that of ethereal sulphate during the 24 hr. following injection; excretions of both forms rapidly regained their normal values. Excretions of neutral sulphur and of glucuronic acid were not significantly affected and no growth retardation was observed.

*Naphthalene.* This hydrocarbon was administered at two dose levels (46 and 86 mg./100 g.). The higher dose led to the death of four of ten rats during the week following the injection, whereas the smaller quantity was well tolerated. In both experiments there was a considerable decrease in the excretion of inorganic sulphate and a moderate rise in the ethereal sulphate in the urine. Excretion of glucuronide was also increased, but the most striking effect was the high output of neutral sulphur. The lower dose of naphthalene did not retard the growth of the rats. The higher dose killed four of ten rats within 72 hr., but, in spite of this, the surviving animals resumed their normal growth rate within 48 hr. after injection.

*Phenanthrene.* Injection of 100 mg./100 g. led to a decrease in inorganic sulphate excretion and increases in ethereal sulphate, neutral sulphur, and glucuronic acid in the urine. A fall in weight during the 24 hr. after injection was succeeded by a period during which growth was less than normal.

*Anthracene.* With 100 mg./100 g. there was no increased ethereal sulphate excretion but a moderately increased output of neutral sulphur and glucuronic acid. The growth rate fell to about 60 % of its control value.

1:2-*Benzanthracene.* 100 mg./100 g. produced a small increase in the excretion levels of neutral sulphur and of glucuronic acid without change in the excretion of ethereal sulphate. The growth retardation was greater than that produced by the same dose of anthracene.

1:2:5:6-*Dibenzanthracene.* The rats tolerated 100 mg./100 g., only two of ten animals dying within 10 days of the injection. During this period no significant change from the normal excretions was observed except a transient fall in inorganic sulphate and a very small rise in neutral sulphur output. Growth of the rats was completely arrested, and the animals maintained a constant weight.

3:4-*Benzpyrene.* 100 mg./100 g. had no effect on the urinary sulphur excretion apart from inducing a fall in the output of inorganic sulphate. The level of excretion of glucuronic acid was higher after injection than during the control period. Complete cessation of growth was observed in these animals after injection.

*Pyrene.* Rats injected with 100 mg./100 g. showed a sharp fall in inorganic sulphate excretion accompanied by relatively large increases in output of ethereal sulphate, neutral sulphur, and glucuronic acid. A considerable fall in body weight occurred during the 4 days immediately following the injection; this decline was then abruptly arrested and was followed by a resumption of growth at 75 % of the normal rate.

*Chrysene.* 100 mg./100 g. produced no significant change in sulphur or glucuronic acid excretion. A fall in body weight occurred immediately after injection and persisted for 48 hr. Growth was then resumed at about half the rate of the pre-injection period.

## DISCUSSION

*The metabolism of aromatic hydrocarbons.* The results may be briefly considered in the light of the experiments are only capable of revealing urinary metabolites of the substances administered when the latter undergo rapid metabolism and excretion. The constant excretion of very small amounts of a

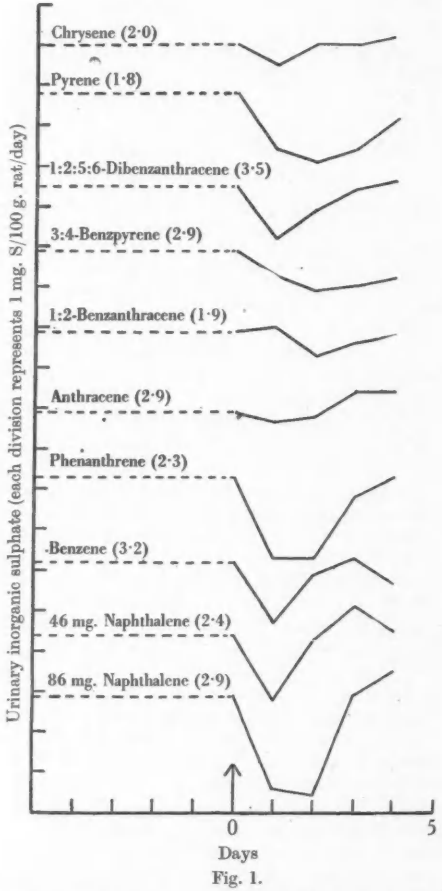


Fig. 1.

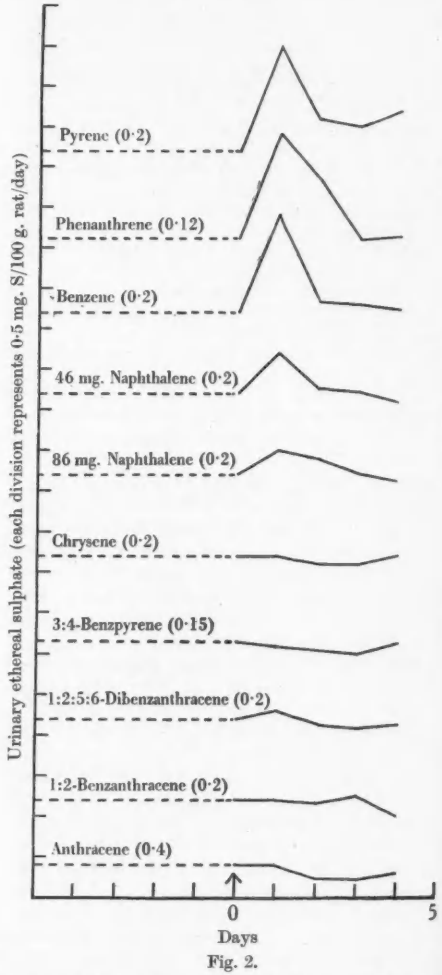


Fig. 2.

Fig. 1. Excretion of inorganic sulphate in the urine of male rats after single intraperitoneal injections of aromatic hydrocarbons. Average value during control period is shown by broken line: the actual values represented by these lines are shown in parentheses. The day of injection is shown by the arrow on the abscissa.

Fig. 2. Excretion of ethereal sulphate in the urine of rats after single intraperitoneal injections of aromatic hydrocarbons. Average value during control period is shown by broken line: the actual values represented by these lines are shown in parentheses. The day of injection is shown by the arrow on the abscissa.

data already available on the metabolism of some of the hydrocarbons used in this work. It must be emphasized that the methods used in the present metabolite of a substance undergoing slow absorption and metabolism would be undetectable, as would the presence of minor metabolites. This is

well shown in the case of benzene, where the rapid formation and excretion of ethereal sulphate is clearly demonstrated (Fig. 2), while the estimations

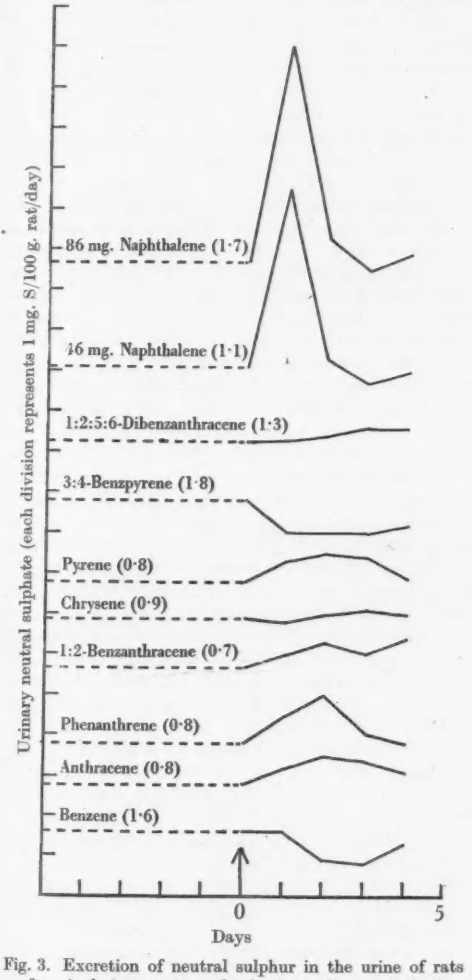


Fig. 3. Excretion of neutral sulphur in the urine of rats after single intraperitoneal injections of aromatic hydrocarbons. Average value during control period is shown by broken line: the actual values represented by these lines are shown in parentheses. The day of injection is shown by the arrow on the abscissa.

of neutral sulphur excretion (Fig. 3) give no indication of the formation of very small quantities of a mercapturic acid which has been isolated by Zbarsky & Young (1943).

The increased excretion of ethereal sulphate and of glucuronide after naphthalene administration, shown in the present experiments, was expected from the fact that in some species naphthalene is known to yield some naphthol (Lesnik, 1888). The most striking feature, however, is the speed and extent of mercapturic acid formation as shown by the large rise in the excretion of neutral sulphur immediately following injection of the hydrocarbon. Bourne & Young (1934) isolated naphthylmercapturic acid from the urine of rabbits fed a diet containing naphthalene, and Stekol (1937) showed that the same substance is a metabolite of this hydrocarbon in the rat.

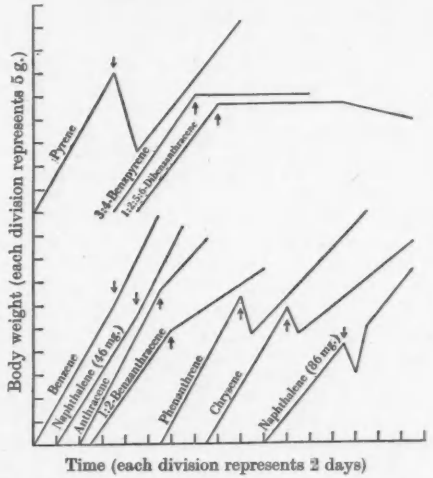


Fig. 4. Effect of single intraperitoneal injections of aromatic hydrocarbons on the rate of growth of male rats. Day of injection of the hydrocarbon is indicated by an arrow.

The increased excretion of ethereal sulphate and of glucuronic acid by the rats injected with anthracene was expected from the results of Boyland & Levi (1935, 1936*a*, *b*). These workers showed that the metabolites of anthracene in the rat included 1:2-dihydroxy-1:2-dihydroanthracene, a glucuronide of this substance, and 1-anthrylmercapturic acid.

Bergel & Pschorr (1903) showed that phenanthrylglucuronic acid probably arises in rabbits treated with phenanthrene. The increased excretion of glucuronide in rats injected with this hydrocarbon might be accounted for by the presence of this metabolite in the urine. The increased output of neutral sulphur points to mercapturic acid formation. A marked difference in the metabolic routes of anthracene and of phenanthrene is indicated by the increased excretion of ethereal sulphate following injection of phenanthrene. This might be

explained by the use of both sulphate and glucuronide in the detoxication of the phenolic group of the phenanthrol, while the alcoholic hydroxyl of 1:2-dihydroxy-1:2-dihydroanthracene is combined exclusively with glucuronic acid.

The results of the present experiments on 1:2-benzanthracene, 1:2:5:6-dibenzanthracene, 3:4-benzpyrene, and chrysene, throw little new light on possible conjugated urinary metabolites of these hydrocarbons. The estimations indicate that the quantities of such metabolites, if they exist at all, are very small, and that attempts at their isolation are unlikely to be profitable unless large amounts of urine from animals receiving high doses of these hydrocarbons are used.

The relatively large response in output of ethereal sulphate, neutral sulphur, and glucuronide, in the rats injected with pyrene, offers more promise for experiments on the isolation of metabolites of this hydrocarbon: this point is being further investigated. During the period of weight loss immediately following injection of this substance it was observed that the animals were suffering from severe diarrhoea. Berenblum & Schoental (1943) identified 3:4-benzpyrene-5:8-quinone in the faeces of rats injected with 3:4-benzpyrene. The diarrhoea caused by injections of pyrene might be produced by excretion into the gut of a pyrene quinone, or a hydroxypyrene quinone, with purgative properties. A preliminary examination of faeces from pyrene-injected rats has given evidence for the presence of one or more pyrene quinone derivatives.

*The connexion between sulphur metabolism and growth retardation.* It is apparent from the curves for average growth rate before and after administration of the hydrocarbons shown in Fig. 4 that three types of response may occur. In the first type the rats continue to show a daily increase in weight even after injection of the hydrocarbon: these daily increases may be equal to those during the control period or they may be less. This type of response is obtained in animals treated with benzene, naphthalene in low doses, anthracene, and 1:2-benzanthracene. In the second type the rats respond to the injection by showing a fall in body weight lasting for several days. After this short period of weight loss, growth is resumed either at a normal or at a retarded rate. Examples of this type occurred in the rats treated with larger doses of naphthalene, phenanthrene, chrysene, and pyrene. The third type encountered in the present experiments may be considered to be a special case of the first type. After injection of 3:4-benzpyrene or of 1:2:5:6-dibenzanthracene there was immediate complete cessation of growth, and the animals maintained a constant weight for a prolonged period.

The immediate, constant, and long-continued reduction in growth rate that Haddow *et al.* (1937) observed with certain carcinogenic hydrocarbons is well shown by the results with the two carcinogens used in the present experiments. The retardation of growth with 3:4-benzpyrene and with 1:2:5:6-dibenzanthracene was greater and persisted longer than that caused by any of the other seven hydrocarbons investigated. According to the views of White & White (1939) this retardation is caused by a lowering of the supply of sulphur-containing amino-acids (essentially cystine or methionine) available for growth, owing to the additional requirements for these acids for metabolism and elimination of the hydrocarbons. An examination of the results for excretion of neutral sulphur shown in Fig. 3 indicates, however, the improbability of such an explanation. Neither with 3:4-benzpyrene nor with 1:2:5:6-dibenzanthracene is there any evidence for the excretion of a mercapturic acid. On the other hand, injection of naphthalene (46 mg./100 g.) is followed by the excretion of large amounts of neutral sulphur, presumably a mercapturic acid, without influencing the rate of growth of the rats. Even with doses of naphthalene that produced toxic symptoms leading to death in some of the animals, and induced a very high neutral sulphur output in the survivors, growth, after a short period of weight loss followed by recovery, continued at an unretarded rate.

The experiments described in this paper demonstrate, therefore, that (*a*) the growth retardation in rats caused by the administration of such hydrocarbons as 3:4-benzpyrene or 1:2:5:6-dibenzanthracene is not accompanied by the excretion of any appreciable amount of sulphur-containing derivatives of these hydrocarbons during the period of retarded growth, and that (*b*) on an adequate diet very considerable demands on the supply of sulphur-containing amino-acids for the purpose of detoxication can be met without retarding the growth of the animals.

These results are, apparently, contradictory to those obtained by White and his colleagues. An examination of the experimental method used by these authors, however, reveals that the results they obtained are by no means unequivocal. Their conclusions were based on the restoration of growth observed on feeding cystine or methionine to rats retarded in growth by the inclusion of hydrocarbons in their diet. Rats on the supplemented diet, but still ingesting hydrocarbon, showed about the same growth rate as when they were fed the unsupplemented diet free from hydrocarbon.

The amount of supplement used by White & White greatly exceeded the theoretical requirement for conversion of all the ingested hydrocarbon into mercapturic acid. Stekol (1943) showed that, in the case of bromobenzene, when due allowance is made for the sulphur required for mercapturic acid

formation, all the remaining sulphur in the diet is available for growth. Hence, in the experiments of White *et al.*, if the growth-inhibitory action of the ingested hydrocarbon has actually been completely annulled by the supplement, then the rats would be expected to show a higher growth rate when receiving the supplemented diet plus hydrocarbon than when on the unsupplemented diet free from hydrocarbon. The excess of the supplement above requirement for detoxication should be available for additional growth. Experiments by the present authors, which will be described in detail elsewhere, have shown that the growth rate of rats on White's basal diet is increased two to four times on addition of cystine or methionine.

Experiments on dimethylaminoazobenzene (White, 1940) and on iodoacetic acid (Stevenson & White, 1940) are open to the same criticism. It appears that, with the methods and criteria employed by these authors, any substance inducing growth inhibition *not* caused by deprivation of sulphur-containing amino-acids would show an apparent connexion with sulphur metabolism.

The results recorded in the present paper, together with a re-examination of the data of White *et al.*, show that the growth retardation produced by such hydrocarbons as 3:4-benzpyrene, methylcholanthrene, and 1:2:5:6-dibenzanthracene, cannot be attributed to the necessity for furnishing sulphur-containing amino-acids for detoxication at the expense of those available for body growth. The growth retardation must be produced by other causes, which are possibly also operative to a smaller extent in the retardation produced by anthracene, 1:2-benzanthracene, phenanthrene, or chrysene (Fig. 4). The cause appears to be more subtle than the mere sulphur deprivation that accounts for the whole of the growth-retarding effects of such mercapturic acid-forming substances as bromobenzene or naphthalene. Further search for the true mechanism is clearly important. An obvious possibility is inhibition of the anterior lobe of the hypophysis with consequent suppression of growth hormone secretion. Such an action is known to occur with oestrogens (Zondek, 1941). Experiments to test this hypothesis are being carried out. Alternatively, interference with any one of a number of vital enzyme systems by the hydrocarbons or their metabolic products could lead to partial or complete cessation of growth in an animal.

The demonstration that sulphur plays no major role in the elimination of such hydrocarbons as 3:4-benzpyrene renders more interesting the experiments of Crabtree (1944) on the inhibition of the carcinogenesis caused by this hydrocarbon. He showed that three classes of substances, which are structurally unrelated but have the common property of fixing labile sulphydryl groups, all produce the effect of checking carcinogenic action. If sulphur is not involved in the elimination of the hydrocarbon this observation points, as Crabtree suggests, to the necessity for combination between the carcinogenic hydrocarbon and sulphydryl at a primary stage in the biological action of the carcinogen. Competition for sulphydryl groups between inhibitor and carcinogen might lead to complete suppression of the carcinogenic change.

## SUMMARY

1. Modifications of the standard procedure for estimating urinary sulphur partition by the volumetric benzidine method are described, and the results of a study of sulphur excretion in normal male rats are given.

2. The excretion of inorganic sulphate, ethereal sulphate, neutral sulphur and glucuronic acid in the urine of male rats injected with benzene, naphthalene, phenanthrene, anthracene, 1:2-benzanthracene, 1:2:5:6:-dibenzanthracene, 3:4-benzpyrene, pyrene, or chrysene was investigated. Observations on the effect of these hydrocarbons on the rate of body growth were also made.

3. No evidence of the excretion of mercapturic acids by rats treated with the carcinogens 3:4-benzpyrene and 1:2:5:6-dibenzanthracene was found. The retardation of growth produced by these hydrocarbons cannot be attributed to deprivation of sulphur-containing amino-acids.

4. A re-examination of the data of White and his colleagues, who arrived at conclusions contrary to the above, indicates that their conclusions are not unequivocal and that their results are in fact compatible with the absence of a connexion between the growth retardation produced by polycyclic hydrocarbons and sulphur metabolism.

We wish to thank Miss P. Hales for her assistance with the animals. One of us (F. L. W.) is indebted to the Sir Halley Stewart Trust for a Fellowship held during this work. Generous grants from the British Empire Cancer Campaign, the Anna Fuller Fund, the Jane Coffin Childs Memorial Fund, and facilities afforded by Imperial Chemical Industries Limited, have also assisted.

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# The Antibacterial Action of 4:6-Dimethoxytoluquinone and its Fate in the Animal Body

By G. E. GLOCK, R. H. THORP (*The Wellcome Foundation Ltd.*), J. UNGAR (*Glaxo Laboratories Ltd.*) and R. WIEN (*May and Baker Ltd.*)

(*Received* 30 *May* 1945)

During the examination of a series of derivatives of fumigatin, 3-hydroxy-4-methoxytoluquinone, Oxford (1942) noted that while toluquinone was much less bacteriostatic, certain other quinones, particularly 4:6-dimethoxytoluquinone (synthesized: Anslow, Ashley & Raistrick, 1938; Aulin & Erdtman, 1938), were more bacteriostatic than fumigatin.

We have investigated the biological properties of the latter quinone for its possible use as a therapeutic agent by local or systemic administration. It was found at an early stage that the compound was inactivated in the presence of blood and it did not give any protection to mice against certain pathogenic organisms. Since we were, however, engaged in research on similar compounds we studied the mode of its deactivation and detoxication in the animal body, as a possible guide for further chemical work in the preparation of more active compounds.

After the completion of the work described in this paper a publication by Barber (1944) appeared on the antibacterial action of 4:6-dimethoxytoluquinone. She found that it had a powerful bacteriostatic action against *Staphylococcus aureus* and *Streptococcus pyogenes* in broth, but owing to its greatly decreased activity in the presence of blood or serum, and to the markedly antileucocytic properties of the compound, it was contra-indicated as a therapeutic agent. Our results agree with these findings.

## EXPERIMENTAL

### Action against bacteria

In vitro *experiments*

The following organisms were used: *Streptococcus pyogenes, Staphylococcus aureus, Diplococcus pneumoniae* (Type 1), *Ps. aeruginosa, Proteus vulgaris, Bech. coli, Cl. perfringens.*

Different types of culture media were used:

(*a*) *Synthetic media.* The synthetic media used were evolved from work described by McIlwain, Fildes, Gladstone & Knight (1939) on the nutrients essential for the growth of bacteria.

For staphylococci a medium was used consisting of: NaCl, 5·0 g.; $K_2HPO_4$, 1·0 g.; $KH_2PO_4$, 1·0 g.; $MgSO_4$, 0·2 g.; $CaCl_2$, 0·1 g.; ammonium lactate, 5·0 g.; acid-hydrolyzed casein, 1·0 g.; distilled water to 1 l.

For streptococci, the following medium was used: $K_2HPO_4$, 0·5 g.: $KH_2PO_4$, 0·5 g.; $MgSO_4.7H_2O$, 0·2 g.; NaCl, 0·1 g.; $FeSO_4.7H_2O$, 0·01 g.; $MnSO_4.4H_2O$, 0·01 g.; glucose, 20·0 g.; sodium acetate, 6·0 g.; adenine, 20 mg.; riboflavin, 0·2 mg.; calcium *dl*-pantothenate, 0·2 mg.; nicotinic acid, 0·2 mg.; pyridoxine, 0·2 mg.; biotin, 0·0001 mg.; distilled water to 1 l. This medium was Seitz-filtered before use, and 1% of acid-hydrolyzed casein was then added. For the other organisms the medium used was as follows: glycerol, 10·0 g.; NaCl, 5·0 g.; $CaCl_2$, 0·1 g.; $MgSO_4$, 0·5 g.; $K_2HPO_4$, 2·0 g.; ammonium lactate, 6·0 g.; acid-hydrolyzed casein, 1·0 g.; distilled water to 1 l.

(*b*) *Papain digest broth*, containing 1% (w/v) glucose.

(*c*) *Papain digest broth*, with the addition of 10% (v/v) normal horse serum.

(*d*) *Normal human defibrinated blood*, used in Fleming's slide-cell technique.

*Testing of anti-bacterial activity.* Serial dilutions of 4:6-dimethoxytoluquinone or the corresponding quinol were added to the tubes containing the media. In making dilutions of 4:6-dimethoxytoluquinone, suitable precautions were taken to prevent oxidation.

The tubes of medium + 4:6-dimethoxyquinone dilutions were inoculated with one standard drop of a saline suspension of a 24 hr. agar culture, containing about $100 \times 10^6$ organisms/ml., and were incubated at 37°. The results were read after 24 and 48 hr. for bacteriostatic effects; the tubes

which showed no growth were subcultured on agar plates, incubated at 37°, and examined after 24 hr. for evidence of bactericidal action.

In Table 1, figures are given for bacteriostatic effects (*a*); the bactericidal effects (*b*) are shown for staphylococci only.

We found that the minimal antibacterial concentration was conditioned partly by the number of organisms in the inoculum (cf. Armstrong, Spink & Kahnke, 1943). Even when the inoculum was standardized, however, variation in the results occurred. An example of this is given by the results obtained with 4:6-dimethoxytoluquinone, the figures in six consecutive experiments being (1) 1 : 100,000, (2) 1 : 100,000, (3) 1 : 200,000, (4) 1 : 40,000, (5) 1 : 80,000 and (6) 1 : 80,000. The bactericidal results showed less variation. It is seen from Table 1 that the quinol was not less active than the quinone. Although serum decreased the activity of the quinone to some extent, there was presumably an interaction with the other constituents of defibrinated blood which rendered the quinone completely inactive.

LOCAL TOLERANCE

*Local toxicity on the conjunctiva.* Two drops of 4:6-dimethoxytoluquinone solution in sterile distilled water at dilutions of 1 : 500 to 1 : 10,000, warmed to 37°, were applied twice daily under the left eyelids of rabbits, leaving the right eyes as controls. No irritation of the conjunctiva was observed after 2 days' treatment.

*Action on tissue culture.* This was tested on growing chick-heart embryo fibroblasts. Dilutions of 4:6-dimethoxytoluquinone were prepared in Tyrode's solution, and then added to a mixture of chick-plasma and embryonic extract on depression slides. Growth of fibroblasts was recorded after 24, 48 and 72 hr. No growth occurred with dilutions up to 1 : 40,000, whereas normal growth of fibroblasts took place in dilutions of 1 : 60,000. It can be inferred that 4:6-dimethoxytoluquinone is comparatively toxic to growing chick-embryo fibroblasts.

*Action on leucocytes.* Human leucocytes in contact for 1 hr. at 37° with dilutions of 4:6-dimethoxytoluquinone up to 1 : 20,000 showed greatly decreased phagocytic activity against a suspension of *Staph. aureus*.

**Table 1.** In vitro *antibacterial activity of* 4:6-*dimethoxytoluquinone and* 4:6-*dimethoxytoluquinol*

(The figures (the mean of several experiments) indicate the minimal effective concentrations: (*a*) bacteriostatic, (*b*) bactericidal (for *Staph. aureus* only).)

| Media | | Organisms: *Staph. aureus* | *Strept. pyogenes* | *Diplococcus pneumoniae* (Type 1) | *Esch. coli* | *Ps. aeruginosa* | *Proteus vulgaris* | *Cl. perfringens* |
|---|---|---|---|---|---|---|---|---|
| **4:6-Dimethoxytoluquinone** | | | | | | | | |
| Glucose broth | (*a*) | 1 : 100,000 | ±1 : 100,000 | 1 : 500,000 | 1 : 5,000 | — | — | <1 : 50,000 |
| | (*b*) | 1 : 20,000 | | | | | | |
| Serum broth | (*a*) | 1 : 20,000 | <1 : 50,000 | 1 : 100,000 | <1 : 5,000 | — | — | 1 : 50,000 |
| Synthetic medium | (*a*) | ±1 : 500,000 | ±1 : 500,000 | — | 1 : 10,000 | <1 : 1,000 | <1 : 1,000 | 1 : 50,000 |
| | (*b*) | <1 : 5,000 | | | | | | |
| Defibrinated blood | (*a*) | <1 : 2,000 | — | — | — | — | — | — |
| **4:6-Dimethoxytoluquinol** | | | | | | | | |
| Glucose broth | (*a*) | 1 : 100,000 | 1 : 500,000 | — | <1 : 5,000 | <1 : 5,000 | <1 : 5,000 | <1 : 50,000 |
| | (*b*) | 1 : 20,000 | | | | | | |
| Serum broth | (*a*) | 1 : 50,000 | 1 : 100,000 | — | <1 : 5,000 | <1 : 5,000 | <1 : 5,000 | — |
| Synthetic medium | (*a*) | 1 : 500,000 | ±1 : 500,000 | — | 1 : 10,000 | ±1 : 5,000 | <1 : 5,000 | 1 : 50,000 |

In vivo *experiments*

(*a*) *Systemic administration.* In mice infected with *Streptococcus pyogenes* or *Staph. aureus*, 4:6-dimethoxytoluquinone or the quinol had no protective effect when administered orally in doses of 0·1 or 0·05 mg./g., respectively, given twice daily, nor in the same doses given once daily by intraperitoneal injection. The treated and the control mice died within 48 hr. after infection.

(*b*) *Local administration.* In mice injected intramuscularly with a suspension of *Cl. perfringens* and 2·5 % (w/v) $CaCl_2$, treatment by local intramuscular injections of 4:6-dimethoxytoluquinone at the site of infection failed to give any protection.

*Local tolerance by intramuscular injection.* A 1 : 1000 solution (0·1 ml.) was injected intramuscularly into the thigh muscles of mice. Macroscopically there was no extensive damage; histological examination showed degeneration of some of the nuclei and swelling of the fibres, but on the whole the damage was small.

*Local effect on wound healing.* The effect of 4:6-dimethoxytoluquinone (5 % (w/w) suspension in an ointment base) was tested on burn wounds produced in guinea-pigs by the method of Ungar & Robinson (1943). Enough ointment was applied daily on vaseline gauze to cover the wound area and the

treatment was continued for 8 days. There was no significant difference in the rate of healing of the treated animals as compared with control animals treated with the ointment base only.

### Toxicity

The acute toxicities were determined in mice by oral administration of the compounds suspended in arachis oil. The L.D.$_{50}$ (mean lethal dose) for 4:6-dimethoxytoluquinone and the quinol were respectively 0·6 and 0·3 mg./g. Absorption after oral administration was rapid, since toxic symptoms appeared within 5–10 min. The main symptom was general depression of the respiration, although the quinol produced an initial stimulation with moderately severe spasmodic convulsions of a mixed type.

A few experiments were made on the acute effects in larger animals. In a cat, anaesthetized with nembutal, 4:6-dimethoxytoluquinone (15 mg./kg.) injected intravenously in saline caused a brief rise in blood pressure and an increase in the depth of respiration. A further 20 mg./kg. caused a similar effect followed by respiratory irregularity. The blood at death was strikingly deoxygenated. Toluquinone itself, which is more toxic, showed certain differences. In a similar animal preparation toluquinone at 14·6 mg./kg. caused a transient rise in blood pressure with an increase in respiratory rate. A further 30 mg./kg. caused a rapid fall in blood pressure with rapid shallow respiration, and death resulted from pulmonary oedema. Toluquinone in its toxic effects resembles benzoquinone (Schulz, 1923), and these experiments extend the analogy to mammals, the earlier literature having dealt only with the effect on frogs. By oral administration to rabbits toluquinone is more toxic than 4:6-dimethoxytoluquinone, and death is always associated with pulmonary damage, which does not occur in animals fed 4:6-dimethoxytoluquinone.

### Metabolism experiments

These experiments were undertaken to ascertain the mode of inactivation, detoxication and fate in the animal body of 4:6-dimethoxytoluquinone; for comparison some experiments were also carried out with toluquinone.

#### *Methods of estimation*

*Estimation of quinones.* The colorimetric methods adopted for the estimation of the quinones were based on the reaction described by Craven (1931) between quinones with a labile hydrogen or halogen atom adjacent to the carbonyl group, and ethyl cyanoacetate. The method of Valeur (1899) was also used in some instances.

(1) *Estimation of* 4:6-*dimethoxytoluquinone.* By modifying Craven's reaction, a satisfactory procedure has been devised for estimating 4:6-dimethoxytoluquinone.

Total 4:6-dimethoxytoluquinone in urine is determined after boiling for 1 hr. under a reflux condenser in 2 N-HCl containing 0·2% $SnCl_2$. After cooling the hydrolysate, ammonium hydroxide solution (50% (v/v) of 0·880 sp. gr. ammonia, in water) is added until all the $Sn(OH)_2$ is precipitated; the quinone is then extracted with chloroform and the mixture centrifuged to remove $Sn(OH)_2$ from the chloroform layer. 2 ml. of the chloroform extract are treated with 0·1 ml. redistilled ethyl cyanoacetate followed by 8 ml. ethanolic ammonia (2 parts of 95% ethanol to 1 part of 0·880 sp. gr. ammonia). The blue colour which develops at room temperature has a generalized absorption above 5900 A; for photoelectric comparison an orange filter transmitting above 5400 A was used. Using a 1 cm. cell, 0·01–0·25 mg. 4:6-dimethoxytoluquinone in 2 ml. chloroform can be estimated. The colour reaches its maximum intensity in 15 min. and is stable for a further 30 min.

4:6-Dimethoxytoluquinone added to urine could be recovered quantitatively using the above procedure. Free 4:6-dimethoxytoluquinone is determined in a chloroform extract of the unhydrolyzed material.

(2) *Estimation of toluquinone.* Total toluquinone is determined after acid hydrolysis as described above for 4:6-dimethoxytoluquinone. The hydrolysate is extracted twice with an equal volume of amyl alcohol and a sample of this extract shaken with an equal volume of saturated $Na_2CO_3$ solution. After centrifuging, the clear amyl alcohol extract is used for colour development. To 2 ml. extract is added 0·5 ml. saturated $KClO_4$ solution (to produce even colour development in the case of hydrolyzed rabbit urine extracts), followed immediately by 8 ml. ethanolic ammonia solution and 0·1 ml. ethyl cyanoacetate. The contents of the tube are mixed after each addition and the reading is made 35–45 min. later. When the amyl alcohol extract is coloured, a blank determination is carried out replacing the ethyl cyanoacetate by water. Toluquinone added to urine can be recovered quantitatively using the above procedure. Free toluquinone is estimated in an amyl alcohol extract of the unhydrolyzed material.

*Inorganic and total sulphates* were determined in urine by the method of Marenzi & Banfi (1939).

*Glucuronic acid.* Glucuronide excretion was usually estimated in the present work by the method of Maughan, Evelyn & Brown (1938), as used by Mozolowski (1940). After the completion of these experiments Hanson, Mills & Williams (1944) published a paper on the estimation of glucuronic acid. The glucuronic acid content of several samples of normal rabbits' urine, and of urine from rabbits fed 4:6-dimethoxytoluquinone, was then estimated, employing the method given by these authors. It was found necessary to increase the time of heating, in the case of the urine containing the conjugated

4:6-dimethoxytoluquinone derivative, to 4 hr. in order to obtain maximum recovery of glucuronic acid. The average recovery of glucuronic acid was 136% of that obtained previously under the conditions of Maughan *et al.* (1938). The value for the glucuronic acid content of the urines after feeding 4:6-dimethoxytoluquinone given in this paper should therefore be increased by approximately 30–40%. Corrected values are included in Table 2.

The standard glucurone was prepared by the method of Williams (1940) from ammonium glucuronide isolated from the urine of rabbits fed *dl*-menthol. The rabbits were only used for short periods (Martin & Rennebaum, 1943).

### *Conjugation of* 4:6-*dimethoxytoluquinone and toluquinone with amino-acids* in vitro

It is known that quinones react with various ammonium salts and amino-acids. Fischer & Schrader (1910) have shown that strong ethanolic solutions of quinones (including toluquinone and benzoquinone) combine with esters of amino-acids to form deep red solutions from which crystals may separate. Under these conditions, two molecules of toluquinone combine with one molecule of amino-acid. Cooper & Nicholas (1927), and Cooper & Haines (1928, 1929) have further shown that dilute aqueous solutions of quinones combine with aqueous solutions of various amino-acids and proteins. The disappearance of free quinone was taken as a criterion of conjugation, using Valeur's method (1899) of estimation, which depends on the liberation of iodine from hydriodic acid. In experiments of this type the ethyl cyanoacetate method is not satisfactory, since the quinol liberated in the reaction also reacts with ethyl cyanoacetate, making the interpretation of the results obscure.

4:6-Dimethoxytoluquinone and the corresponding quinol were incubated at 37° at pH 7·3 for varying lengths of time up to 4 days with glycine, alanine, and albumen, altering the concentration of the reagents. On estimation of the free quinone by Valeur's method, no evidence of reaction was obtained.

In the case of toluquinone, equal volumes of 0·2% toluquinone and glycine were allowed to react at room temperature in the dark and samples removed at intervals and analyzed for free quinone as above. The free COOH group of any conjugated amino-acid was also determined by titration with 0·01N-NaOH. The reaction mixture turned an intense red. In a typical experiment, after allowing the reaction to proceed for 43 hr., 34% of the free toluquinone had disappeared. The results of the acidimetric titration indicated that glycine had combined with toluquinone in the proportion of 1·2 molecules of glycine to 2 molecules of toluquinone. This is in approximate agreement with Fischer & Schrader (1910).

### *Disappearance of* 4:6-*dimethoxytoluquinone added to blood* in vitro

The observations by Austin & Drabkin (1935) and Scudi (1942), that quinones in general cause methaemoglobin formation in laked blood, have been confirmed for 4:6-dimethoxytoluquinone, but this is of little significance *in vivo*. Even when large doses of the drug are fed, its low solubility and rapid excretion prevent detectable amounts of methaemoglobin from appearing in the systemic circulation. When citrated rabbit blood was incubated at 37° with one-tenth of its volume of a warm saturated solution of 4:6-dimethoxytoluquinone in saline, the proportions of free quinone recovered from the blood at 2, 75, 130 and 1320 min. were, respectively, 79, 53, 24 and 0%. No quinone could be recovered after acid hydrolysis. The disappearance of free quinone was greater when incubated with whole blood or suspended washed red cells than with plasma, e.g. after 2 hr. incubation with one-tenth of the volume of saturated 4:6-dimethoxytoluquinone 29% free quinone was recovered from a suspension of washed rabbit red cells and 63% from the corresponding plasma. Again no quinone was recovered after acid hydrolysis. By modifying the hydrolysis procedure, a small proportion of the conjugated 4:6-dimethoxytoluquinone could be recovered from the blood previously incubated with 4:6-dimethoxytoluquinone until no free quinone was left. A maximum recovery of 20% was obtained by increasing the concentration of $SnCl_2$ tenfold.

A mixture of citrated rabbit blood, hydrochloric acid containing $SnCl_2$ and saturated 4:6-dimethoxytoluquinone solution, extracted immediately with chloroform, gave only 50% recovery of free quinone. This indicated that 4:6-dimethoxytoluquinone probably very readily forms an unhydrolyzable complex with acid haematin; for this reason further experiments in this direction were not attempted.

### *Feeding experiments on rabbits*

Preliminary qualitative tests suggested that after feeding 4:6-dimethoxytoluquinone to rabbits a considerable amount of the compound was excreted conjugated with glucuronic acid. This is perhaps due, among other things, to the fact that in the organism the compound probably exists as the quinol because of its high oxidation reduction potential (Page & Robinson, 1943). The combined form could be detected in the urine within 30 min. of feeding the compound.

The rabbits were housed in metabolism cages and the urine and faeces collected separately. The diet consisted of 100 g. of washed sugar-beet pulp, bran and oats (4 : 1 : 1)

Table 2. *The effect of oral administration of 4:6-dimethoxytoluquinone on the urinary excretion of glucuronide and quinone by rabbits*

(C1–C4 = pre-experimental period. TQ1–TQ4 = experimental period. S1–S4 = control period with food intake corresponding to TQ1–TQ4. A = values obtained using method of Maughan *et al.* (1938). B = corrected values using factor of 1·36 obtained after 4 hr. hydrolysis.)

| | Dose of 4:6-dimethoxytoluquinone | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 700 mg. | | | | 650 mg. | | | | 625 mg. | | | |
| | | Glucuronide* (mg.) | | | | Glucuronide* (mg.) | | | | Glucuronide* (mg.) | | |
| 24 hr. periods | Urine volume (ml.) | A | B | Quinone (mg.) | Urine volume (ml.) | A | B | Quinone (mg.) | Urine volume (ml.) | A | B | Quinone (mg.) |
| C1 | 250 | 165 | — | — | 350 | 235 | — | — | 280 | — | — | — |
| C2 | 220 | 94 | — | — | 310 | 124 | — | — | 310 | 230 | — | — |
| C3 | 250 | 162 | — | — | 330 | 202 | — | — | 310 | 205 | — | — |
| C4 | 270 | 117 | — | — | 290 | 127 | — | — | 320 | 212 | — | — |
| TQ1 | 60 | 261 | 355 | 325 | 70 | 268 | 365 | 375 | 190 | 320 | 436 | 277 |
| TQ2 | — | — | — | — | — | — | — | — | 240 | 180 | 245 | 110 |
| TQ3 | 200 | 205 | 279 | 29 | 275 | 242 | 329 | 50 | 330 | 270 | — | 0 |
| TQ4 | 190 | 74 | — | 0 | 200 | 63 | — | 0 | — | — | — | — |
| S1 | 75 | 63 | — | 0 | 118 | 82 | — | 0 | 120 | 117 | — | 0 |
| S2 | — | — | — | — | — | — | — | — | 240 | 240 | — | 0 |
| S3 | 160 | 109 | — | 0 | 175 | 110 | — | 0 | 200 | 200 | — | 0 |
| S4 | 130 | 89 | — | 0 | 200 | 115 | — | 0 | — | — | — | — |

* Expressed as glucurone.

wetted with 300 ml. water, and 150 g. fresh cabbage daily. 4:6-Dimethoxytoluquinone (250 mg./kg. body wt.) suspended in warm arachis oil (in which it is partially soluble) or toluquinone (70 mg./kg.) suspended similarly, was fed by stomach-tube as the experiment required. Larger doses were often fatal. The urine was preserved with chloroform or toluene, introduced into the collecting vessel, to prevent bacterial hydrolysis of the conjugated excretion product.

Table 2 shows the total hydrolyzable quinone excreted in rabbits. No free 4:6-dimethoxytoluquinone or toluquinone was excreted. In the case of 4:6-dimethoxytoluquinone an average of 54% of the drug was recovered in a conjugated acid hydrolyzable form, while with toluquinone the average was 46%. We have not been able to account for the rest of the ingested quinones. No 4:6-dimethoxytoluquinone, either free or combined, detectable by our conditions of hydrolysis, is eliminated in the faeces.

In addition to the excretion of conjugated acid-hydrolyzable quinones, the excretion of glucuronic acid and inorganic and total sulphates has been followed. In the case of toluquinone, although it would have been desirable to follow the excretion of amino-acids, this was not possible, since even if all the toluquinone given had been excreted combined with amino-acid, no significant increase in the total excretion of amino-acid would have been observed.

The urine was collected for 24 hr. periods over a pre-experimental period of 4 days and for a further 4 days after the compound was given. Both compounds caused a marked decrease in the food intake, accompanied by a corresponding decrease in urinary volume. This in itself affected considerably the level of glucuronic acid excretion. In some experiments the urine was collected for a further control period of 4 days, the food intake being adjusted to correspond with that over the experimental period. There was no increase in the excretion of ethereal sulphate after ingestion of 4:6-dimethoxytoluquinone or toluquinone; a slight increase would not have been significant. Table 2, however, shows the increased excretion of glucuronide and conjugated acid-hydrolyzable quinone after feeding 4:6-dimethoxytoluquinone. The absolute increase in glucuronide excretion cannot be given since the basal daily excretion varies within considerable limits. If we suppose, however, that 4:6-dimethoxytoluquinone combines with glucuronic acid in molecular proportions, it is highly probable that the whole of the 4:6-dimethoxytoluquinone appears in the urine as glucuronide.

Table 3. *The effect of oral administration of toluquinone on the urinary glucuronide and quinone excretion by a rabbit*

(The animal received a dose of 65 mg. toluquinone.)

(C1–C4, pre-experimental period; TQ1–TQ3, experimental period; S1–S3, control period with food intake corresponding to TQ1–TQ3.)

| 24 hr. periods | Urine volume (ml.) | Glucuronide* excreted (mg.) | Quinone excreted (mg.) |
|---|---|---|---|
| C1 | 230 | 208 | — |
| C2 | 270 | 174 | — |
| C3 | 270 | 194 | — |
| C4 | 320 | 204 | — |
| TQ1 | 150 | 150 | 50 |
| TQ2 | 130 | 135 | 20 |
| TQ3 | 170 | 125 | 0 |
| S1 | 270 | 196 | — |
| S2 | 150 | 140 | — |
| S3 | 120 | 162 | — |

* Expressed as glucurone.

The corresponding experiment with toluquinone itself (Table 3) showed no increase in glucuronide excretion, so that, unlike 4:6-dimethoxytoluquinone, toluquinone is presumably not detoxicated by conjugation with glucuronic acid.

## DISCUSSION

Although 4:6-dimethoxytoluquinone is highly bacteriostatic *in vitro*, its activity is decreased or abolished in the presence of serum and blood, and it is inactive *in vivo*. It is comparatively toxic to leucocytes and growing chick embryo fibroblasts and is unlikely to be of value as a therapeutic agent. One of our colleagues, Dr Harrison, has found it to be fungicidal against *Actinomyces praetorianus* and *Trichophyton lacticolor* in concentrations of about 1 : 100,000, and it may possibly have some application in this field.

Since compounds of this type are common products of mould metabolism it is of interest to note that 4:6-dimethoxytoluquinone is very readily conjugated with glucuronic acid in the animal and that an adequate blood concentration cannot be obtained. Most of the ingested quinone can be recovered from the urine in a form mainly or entirely glucuronide. This has no antibacterial activity and is detectable in the urine within 30 min. of feeding the compound. Neither free nor acid-hydrolyzable conjugated quinone can be recovered from the faeces. The free quinone disappears fairly rapidly when it is incubated with whole blood *in vitro*, but the speed of disappearance is insufficient for this combination in the blood to assume significant proportions in the animal body.

## SUMMARY

1. The *in vitro* antibacterial activities of 4:6-dimethoxytoluquinone and 4:6-dimethoxytoluquinol against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Diplococcus pneumoniae* (Type 1), *Ps. aeruginosa*, *Proteus vulgaris*, *Esch. coli* and *Cl. perfringens* have been investigated. Both compounds were found to be highly bacteriostatic against Gram-positive organisms.

2. The bacteriostatic activity of 4:6-dimethoxytoluquinone was decreased in the presence of serum and abolished in the presence of blood.

3. Oral administration of the quinone gave no protection in mice infected with *Streptococcus pyogenes* or *Staphylococcus aureus*, and local injections were ineffective against experimental *Cl. perfringens* infections.

4. In tissue-culture experiments, the quinone, in a concentration of 1 : 40,000, inhibited the growth of chick-heart embryo fibroblasts. It also decreased the phagocytic action of leucocytes.

5. The quinone is rapidly conjugated with glucuronic acid when administered orally to rabbits, and appears in the urine in this form within 30 min. of feeding; the glucuronide has no antibacterial activity.

6. The fate of 4:6-dimethoxytoluquinone in the body is different from that of toluquinone itself, which is detoxicated by conjugation with amino-acids and not with glucuronic acid.

7. On account of its general toxic reactions, its inactivation in the presence of blood and rapid conjugation with glucuronic acid in the animal body, 4:6-dimethoxytoluquinone presumably has little therapeutic value.

The work described in this paper was carried out as part of a programme of the Therapeutic Research Corporation of Great Britain Ltd., to which acknowledgements are given. We are indebted to Dr Ashley for the supply of the quinones used.

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# The Production of Penicillin Using Fractions Obtained from Aqueous Extracts of Pea (*Pisum sativum*)

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(*Received* 31 *May* 1945)

Aqueous extracts of air-dried ground peas can provide a good medium for the growth and production of penicillin by *Penicillium notatum* (Cook & Tulloch, 1944). The experiments to be described were carried out to determine what constituents of the pea were responsible for this effect. Although other vegetable extracts give a similar effect the pea has been chosen as it is a readily available material and one on which methods may be standardized.

## EXPERIMENTAL

*Raw material.* Dried green peas obtained from a local seedsman were used; the variety of seed was found not to affect the results. The dried peas were ground in a coffee-mill and the pea-meal kept in a cool place. No deterioration in the activity of extracts was found after storage. Dundee mains water was used to prepare extracts; distilled water was used to reconstitute evaporated fractions.

*Fractionation of the pea extract.* Both hot and cold water extracts were prepared and fractionated as shown in Fig. 1.

### *Chemical methods of analysis used*

*Dry weight.* Materials were dried to constant weight in an air oven at 100°. *Ash.* The dried material was ashed at 1000° in an electric furnace.

*Estimation of mineral constituents.* After the ash was dissolved in HCl, K was estimated using the Na salt of 6-chloro-5-nitrotoluene-3-sulphonic acid (Davies & Davies, 1923). Ca was estimated as oxalate; $P_2O_5$ as the molybdate complex; $SO_4^{--}$ as $BaSO_4$ (Cumming & Kay, 1942).

*Nitrogen.* (i) *Total-N.* The micro-Kjeldahl method was used with the digestion mixture recommended by Chibnall, Rees & Williams (1943). Triplicate determinations were made, each sample being digested for 4 hr. after clearing. (ii) *Non-protein N* was determined in the 15% (w/v) trichloroacetic acid filtrate. (iii) *Amide-N* was determined after hydrolysis with 5% HCl. (iv) *Amino-N* by the Sorensen formol titration method. (v) *Basic-N* was determined on the phosphotungstic acid precipitate.

*Carbohydrate.* The amount of carbohydrate present, before and after hydrolysis by 2N-HCl and after fermentation with yeast, was estimated as glucose by the Hagedorn and Jensen method. Although the carbohydrates are not only glucose, nor convertible into glucose, they are expressed as such as a convenient basis of comparison.

*Galactose.* The mucic acid method of Van der Haar (1917) was used.

### *Biological methods*

*Preparation of media.* Although the pea extracts alone will support growth and permit some penicillin formation (Cook & Tulloch, 1944), this simple medium by itself does not always give consistent results. It has now been found that both growth and penicillin formation may be stabilized by the addition of certain other substances. The most interesting of these is NaCl, the presence of which enhances the yield of penicillin. Other salts ($NaNO_3$, $KH_2PO_4$, $MgSO_4.7H_2O$) also appear to have some effect in stabilizing the yield. The addition of lactose also exerts a similar though less marked effect and is now included in the medium. A basal medium is made up to contain (as g./l.): NaCl, 10; $NaNO_3$, 3; $KH_2PO_4$, 0·5; $MgSO_4.7H_2O$, 0·25 and lactose, 30. Fractions of material from the pea are added to the basal medium approximately in the proportion in which they occur in a '10% extract' (see Fig. 1).

As some of these fractions were added as concentrates the volume was made up with distilled water to that corresponding to the original. In general, no adjustment of pH was made, the values usually ranging between pH 5–6. In cases where ashed or acid fractions were added the pH was adjusted to 5·5. The basal medium itself did not support growth. We are not completely satisfied that this basal medium is the most suitable, but until further work has been done it has been convenient. The basal medium, with added fractions from the pea extract, was made up to 40 ml. and all experiments have been carried out using this amount in 250 ml. conical Pyrex flasks. The flasks containing the complete medium were autoclaved for 15 min. at 15 lb. pressure.

*Seeding culture.* The flasks were seeded by means of a platinum loop from cultures of *P. notatum* grown on Sabouraud's medium, upon which the stock cultures were kept. The strain used throughout was Peoria 1249 B 21. After seeding, the flasks were incubated at 24° ± 1°.

*pH.* The pH of the culture medium was determined from the seventh day of growth onwards using a spiral glass electrode and valve potentiometer (Unicam Instruments Ltd.).

*Measurement of mould growth.* At the end of an experiment the fluid was decanted off, the mycelial felt drained, pressed between absorbent papers until practically dry and weighed.

*Penicillin assay.* The activity has been determined by Brodie's method (1945). We should like to stress that, although the error of this method may be high (±25%), our results are comparative. We have repeated with different batches of material all our series of experiments and no marked variation in the results has been observed.

## RESULTS

Typical results obtained for growth and penicillin production obtained when various fractions of material from hot-water extracts of pea meal were tested are shown in Table 1. Similar results were obtained using cold-water extracts; in general growth and penicillin production reached a peak earlier with the latter although the maximum yields were the same as those given by hot-water extracts. residue by itself hardly supported growth and the yield of penicillin was low (2). Although not shown in Table 1, further extracts made from the pea residue (fraction II) were relatively inactive. Using 80% ethanol as a fractionating agent a fair separation of active constituents was obtained. Thus fraction V had an activity much greater than fraction VI; but on combining these two fractions the activity was greater than that of either fraction alone (4, 9 and 13). This indicated that there were

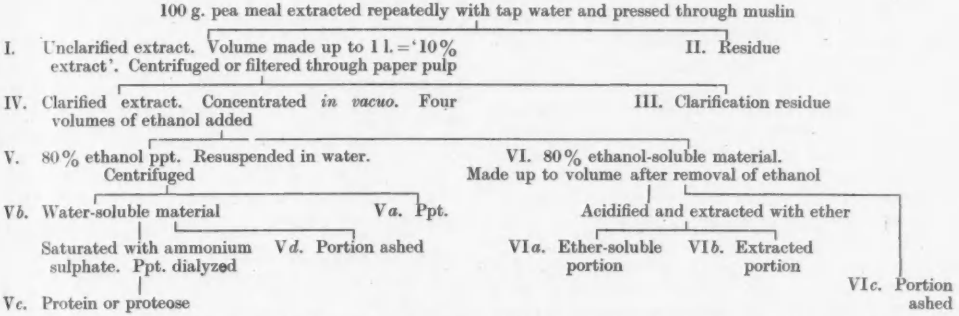
100 g. pea meal extracted repeatedly with tap water and pressed through muslin
I. Unclarified extract. Volume made up to 1 l. = '10% extract'. Centrifuged or filtered through paper pulp
II. Residue
IV. Clarified extract. Concentrated in vacuo. Four volumes of ethanol added
III. Clarification residue
V. 80% ethanol ppt. Resuspended in water. Centrifuged
VI. 80% ethanol-soluble material. Made up to volume after removal of ethanol
Vb. Water-soluble material
Va. Ppt.
Acidified and extracted with ether
Saturated with ammonium sulphate. Ppt. dialyzed
Vd. Portion ashed
VIa. Ether-soluble portion
VIb. Extracted portion
VIc. Portion ashed
Vc. Protein or proteose

Fig. 1. Scheme of fractionation of pea meal to separate material active in stimulating penicillin production.

Table 1. *The growth-promoting and penicillin-producing activities of pea-meal fractions shown in Fig.* 1

(Text number (column 1) is referred to in text. The fraction numbers (column 2) are those used in Fig. 1.)

| Text no. | Fraction in Fig. 1 | Dry matter (g./100 ml.) | Ash (g./100 ml.) | Total N (g./100 ml.) | Wet wt. of mycelium (g.) | Penicillin production units/ml. days in ( ) | pH* |
|---|---|---|---|---|---|---|---|
| 1 | I | 4·0 | 0·27 | 0·19 | 2·27 | 225 (12) | 8·4 (14) |
| 2 | III | 2·0 | 0·02 | 0·09 | 0·85 | 25 (12) | 7·7 (12) |
| 3 | IV | 2·0 | 0·26 | 0·09 | 1·92 | 225 (12) | 8·2 (14) |
| 4 | V | 1·2 | 0·19 | 0·05 | 2·03 | 175 (12) | 7·9 (14) |
| 5 | V*a* | 0·2 | 0·02 | 0·01 | 1·04 | 20 (12) | 7·5 (12) |
| 6 | V*b* | 1·03 | 0·18 | 0·05 | 1·60 | 140 (12) | 7·4 (12) |
| 7 | V*c* | 0·31 | 0·05 | 0·03 | 1·42 | 0 | — |
| 8 | V*d* | 0·18 | 0·18 | 0 | 0 | 0 | — |
| 9 | VI | 1·0 | 0·06 | 0·02 | 1·27 | 15 (12) | 7·4 (14) |
| 10 | VI*a* | 0·2 | 0 | 0 | 0 | 0 | — |
| 11 | VI*b* | 0·8 | 0·06 | 0·02 | 1·73 | 15 (12) | 7·5 (12) |
| 12 | VI*c* | 0·06 | 0·06 | 0 | 0 | 0 | — |
| 13 | V + VI | 2·2 | 0·25 | 0·07 | 1·99 | 225 (12) | 8·5 (14) |
| 14 | V*b* + VI | 2·03 | 0·24 | 0·07 | 2·22 | 180 (12) | 7·6 (12) |
| 15 | V*c* + VI | 1·31 | 0·11 | 0·05 | 0·84 | 20 (10) | 7·7 (10) |
| 16 | V*d* + VI | 1·18 | 0·24 | 0·02 | 1·08 | 80 (12) | 7·9 (12) |

* Time at which determination was made (days) given in parentheses.

In the following text Roman figures and letters refer to fractions in Fig. 1; the arabic numerals in brackets refer to Table 1, where these text-numbers in column 1 refer to the activities, etc., of the corresponding fractions or combinations of fractions.

The results show that the clarification process, in which the dry matter and N content were reduced by half, had no effect on growth and penicillin production (Table 1; text nos. 1 and 3). The clarification essential constituents in fraction VI. Further fractionation of fraction V showed that fraction V*a*, which consisted mainly of starch and insoluble protein, could be removed without affecting the yield, although it had some slight activity (5). Fraction V*b* alone had marked activity; this was further increased by the addition of fraction VI (6 and 14). Fraction V*c* was inactive; even tested in presence of fraction VI the activity was not greater than that

of VI alone (7 and 15). Fraction V*d* alone was inactive, but fractions (V*d*+VI) together gave five times the value for either fraction alone (8, 9 and 16). These results indicated that in fraction V important constituents were contained in the ash, but that the protein exerted no marked effect. Further fractionation of fraction VI showed that fraction VI*a* did not support growth and that fraction VI*b* behaved like fraction VI (10 and 11). Fraction VI*c* did not support growth (12), nor did its addition to fraction V*b* increase the activity of the latter. It appears that in fraction VI ether-soluble acids and mineral constituents of the ash are unimportant.

Analyses of different batches of the two most active fractions (V*b* and VI) show that although there are variations in their composition certain constituents are always present. Typical analyses are presented below.

*Analyses of fraction V b*

The solution was slightly dextrorotatory. The carbohydrate was non-reducing and gave a blue colour with iodine. That it contained carbohydrate other than starch was suggested by the formation of mucic acid after treatment with nitric acid. The mucic acid was identified by: m.p. 204°, mixed m.p. 205° (slow heating); equivalent 105 (theory 105). The galactose thus assumed to be present is apparently in the form of the actual sugar in combination, as tests for uronic acids were negative. The amount of carbohydrate present (as glucose) after acid hydrolysis was 40 % of the dry weight, 5 % of which was galactose.

*Nitrogen.* The total N was 8 % of the dry weight. The distribution of N (as % total) was: non-protein-N, 71; amino-N, 8; amide-N, 10; basic-N, 73. The small amount of protein or proteose present (fraction V*c*) was precipitated by protein reagents and gave the usual colour tests with the biuret, xantho-proteic, Millon and glyoxylic reagents. The ether-extractable material obtained by extracting the acidified fraction was 28 % of the dry weight. This material was dark in colour, but distinct droplets of an oily substance could be seen. This oil was soluble in chloroform and had an equivalent of 234. *Ash.* This was 14 % of the dry weight. Analysis of the ash gave the following values (as % total ash): $K_2O$, 24; CaO, 5; $P_2O_5$, 15; $SO_4^=$, 17. In addition to these, trace elements were found in considerable variety, the following spectrographic analysis of the ash being obtained by Dr R. Mitchell: Mo, 44; Ni, 10; Sn, 30; Pb, 200; Mn, 1000; Rb, 1000; Ba, 200; Sr, 200; Cr, 10 p.p.m. and Cu, 1 %. The Mo determination was made quantitatively but the other values are approximations. The Mo content of fraction V*b* itself was 4·5 p.p.m.

*Analysis of fraction VI*

The solution was slightly dextrorotatory. The carbohydrate estimated as glucose before acid hydrolysis was 16 % of the dry weight and after hydrolysis with 2N-HCl it was 56 %; galactose was 30 %. After acid hydrolysis the following values (as % total carbohydrate) were found: fermentable sugar 60, non-fermentable sugar 40. Tests indicated the presence of glucose and fructose. Ether extraction after acidification yielded 2 % of material and had an equivalent of 250.

*Nitrogen.* Total N: 4 %; the distribution of N (as % total) was: non-protein-N, 100; amino-N, 16; amide-N, 11; basic-N, 59. *Ash.* 5 %, of which 50 % was $K_2O$, 20 % $P_2O_5$. Tests for phenolic compounds were strongly positive.

*Trace elements*

As mentioned above, the mineral portion of the ethanol-precipitable fraction (V) appeared to be important. The migration of the trace elements from the medium into the mould mycelium has been followed by Dr Mitchell. In Table 2 are shown semi-quantitatively the distribution of trace elements in the pea-medium, in Coghill corn-steep medium, and in the mycelium. The result for Sn is from a batch of Coghill medium prepared by Prof. J. Cruickshank; our Coghill medium showed only traces of this metal.

Table 2. *The migration of trace elements from media to mycelium*

Spectrographic analysis of the ashes of pea medium and Coghill medium before growth, and of mycelium of *P. notatum* grown thereon.

+, + +, etc. indicate relative amounts present in the ash; − indicates that the presence of the element was not demonstrable. The sign ⟶ indicates that the element migrates into the mycelium.

| | Pea medium before growth | | Mycelium | Coghill medium before growth | | Mycelium |
|---|---|---|---|---|---|---|
| Ag | + | | | − | | |
| Pb | + + | ⟶ | + | + | ⟶ | + |
| Sn* | − | | | + + + | ⟶ | + + + |
| Mo | + + | ⟶ | + + | + | ⟶ | + |
| Cr | + | | | − | | |
| Ni | + + + | ⟶ | + | + + + | ⟶ | + |
| Co | − | | | − | | |
| Zn | + | ⟶ | + | + | ⟶ | + + |
| V | − | | | + | ⟶ | + |
| Ba | + + | ⟶ | + | + | ⟶ | + |
| Sr | + + | ⟶ | + | + | ⟶ | + |
| Li | − | | | − | | |
| Rb | + + | ⟶ | + | + + | ⟶ | + |

* The result for Sn is for a batch of medium made by Prof. J. Cruickshank. Our medium gave Sn +.

## DISCUSSION

Precipitation with ethanol was used to obtain fractions by a mild method. With hot-water extracts a large amount of material, containing much mineral matter and two-thirds of the total N, was precipitated by ethanol. From cold-water extracts ethanol precipitated much less material. The precipitated material contains carbohydrate, ether-soluble compounds, N-containing compounds and mineral constituents.

The carbohydrate consists of starch and a carbohydrate apparently containing galactose. The presence of galactose in hemicellulose obtained from ungerminated peas was shown by Schulze, Steiger & Maxwell (1890) and Schulze (1892). The ether-soluble matter is present in large amount. The presence of lecithin in the pea has been demonstrated by Winterstein & Hiesstand (1908). The distribution of N shows that a large proportion of it is present in compounds of a basic nature. The trace elements of the large mineral portion of the fraction are of interest, particularly the Mo, previously demonstrated by Ter Meulen (1932) and by Bertrand (1939).

In the ethanol-soluble fraction carbohydrate forms a large proportion of the dry weight but the ether-soluble portion is small in amount. The N is apparently present mainly in compounds of a basic nature. The inorganic moiety appears to consist mainly of potassium phosphate and is not important in penicillin production.

Although both the ethanol-soluble and ethanol-insoluble fractions support growth of the mould, it is the ethanol-precipitable fraction which stimulates penicillin production. There are, however, components in the ethanol-soluble fraction which increase its activity, particularly if the extracts have been prepared with cold water or if germinated or fresh pea extracts are used.

Routine determinations of pH showed that although an increase in pH of the medium was often associated with increased penicillin production this is not always so. A marked increase in pH was always shown early in media in which the protein and starch contents were high, as for example, in unclarified extracts.

## SUMMARY

1. A scheme of preliminary fractionation of aqueous extracts of ground dried peas to separate the constituents responsible for penicillin production is described.

2. 80% ethanol precipitates a fraction which is very active. The ethanol-soluble portion, although it supports growth, has only slight activity in affecting penicillin production, but in the presence of the precipitable fraction increases the latter's activity.

3. Preliminary analyses of the 80% ethanol-soluble and ethanol-insoluble fractions are given.

4. The inorganic constituents of the precipitable fraction are important. The migration of certain trace elements from the medium to the mycelium has been demonstrated.

We are indebted to the Medical Research Council for a personal grant to M. B. B., and to the Helen S. Johnston Memorial Trust for financial assistance. Dr R. G. Tomkins suggested the use of large amounts of NaCl in the medium, and Dr R. Mitchell of the Macaulay Institute for Soil Research kindly carried out the spectrographic analyses.

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# The Glucose Metabolism *in vitro* of *Trypanosoma rhodesiense*

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(*Received* 6 *June* 1945)

It was first shown by Biot, Biot & Richard (1911) that glucose is favourable to the continued life and motility *in vitro* of the rat-trypanosome *T. lewisi*; this effect of glucose has been confirmed by many authors using different species of trypanosomes. Kudicke & Evers (1924) showed that other sugars and glycerol could be used to maintain *T. brucei* in culture media. The rates of glucose decomposition, oxygen consumption, and acid production by certain trypanosomes have been determined (Nauss &

Yorke, 1911; Fennyvessy & Reiner, 1924, 1928; Fennyvessy, 1926; Reiner, Smythe & Pedlow, 1936; Christophers & Fulton, 1938). The mechanism of breakdown of glucose and glycerol was studied by Reiner *et al.* (1936) in the case of *T. equiperdum* and *T. lewisi*, and the metabolic products described; the role of $CO_2$ in the glucose metabolism of *T. lewisi* has been investigated more recently by Searle & Reiner (1940, 1941).

In the case of a normal and an arsenic-resistant strain of *T. rhodesiense* kept in buffered medium at 37°, Christophers & Fulton (1938) found that the ratio of molecules of glucose decomposed to oxygen consumed approached unity; the ratio of equivalents of acid formed to oxygen consumed was approximately 2, so that for one molecule of glucose used, two equivalents of acid were formed. In the present experiments, the nature of the glucose metabolic products of the normal strain of *T. rhodesiense* has been determined. The quantitative aspect of this investigation suffered through enemy action.

## MATERIALS AND METHODS

The strain of *T. rhodesiense* studied was that used in earlier work (Christophers & Fulton, 1938). To obtain live trypanosomes in quantity, mice were infected intraperitoneally; when their blood was swarming with trypanosomes, cardiac puncture was performed aseptically under anaesthesia and as much blood as possible withdrawn. The blood was mixed in centrifuge tubes with about one-third of its volume of 2% sodium citrate containing 0·1% glucose. The pooled blood from a number of animals was centrifuged for 10 min. at 1700 r.p.m. The plasma layer was removed, and then the layer of trypanosomes was pipetted off from above the red cell layer. After suspension of the trypanosome mass in glucose-Ringer solution, the whole was recentrifuged at the same speed for 5 min. and the process of washing repeated. The washed trypanosomes, now free from red cells, were removed in volumes of 1 ml. at a time, and added to 100 ml. of a mixture of 1 volume of M/15 phosphate buffer (pH 7·4) and 1 volume of Ringer's solution containing 0·6% glucose. This investigation was carried out on a very large scale and many hundreds of mice were used.

In one series of experiments 3% (v/v) of horse serum was added to the medium, since it, like rabbit serum, appeared to exercise a favourable influence on the survival of the trypanosomes as judged by the results of respiration and the yield of metabolic products, which was much larger when serum was present. In another series of experiments, serum was not added.

## RESULTS

The dilute suspensions of trypanosomes were kept in sterile flasks at room temperature with frequent shaking. Trypanosomes can be kept alive for long periods under these conditions, but at the end of 6 hr., when the medium had become strongly acid, proteins were precipitated by the addition of $\frac{2}{3}$N-$H_2SO_4$ and an equal volume of 10% sodium tungstate. The precipitate was removed by centrifugation and examined for the presence of metabolic products, with negative results.

*Detection of pyruvic acid.* No aldehyde was detected in the medium before or after precipitation of protein. Tests were made for the presence of ketones by adding 2:4-dinitrophenylhydrazine in 2N-HCl to the protein-free metabolic solution, when a yellow amorphous precipitate appeared after a short time; it was collected by centrifugation. The product separated in microcrystalline form on solution in a large volume of chloroform followed by concentration, after filtration from a little insoluble material. The product was then dissolved in ethyl acetate, extracted with sodium carbonate solution, and recovered by acidification. The deep yellow leaflets (found: C, 40·3; H, 3·0; N, 20·3%. Calc. for $C_9H_8O_6N_4$: C, 40·3; H, 3·0; N, 20·8%) were identical (m.p. and mixed m.p. 213° decomp.; cf. Allen, 1930) with authentic pyruvic acid dinitrophenylhydrazone.

*Detection of ethanol.* Four lots of 600 ml. of the protein-free metabolic solution were slowly distilled using a very efficient fractionation column; 50 ml. of distillate were collected from each lot up to 100° and gave a positive iodoform reaction. The resulting 200 ml. of distillate were redistilled, and 2 ml. of a liquid boiling at approximately 78° collected. It was identified as ethanol by various tests; the *p*-nitrobenzoate melted at 56·5° alone or mixed with an authentic specimen.

*Detection of acetic and formic acids.* The remainder of the protein-free solution was concentrated at 16–20 mm. Hg pressure at a temperature not exceeding 40°. A trap containing 0·1N-NaOH was inserted to catch any volatile acid which distilled. The contents of the trap were allowed to evaporate in air, yielding *c.* 4·5 g. of mixed salts, including some carbonate. Part of this material was dissolved in dilute sulphuric acid and fractionally extracted, first with ether-light petroleum and then with ether. The first small fraction (containing 3–4% of the total organic acid) gave no evidently homogeneous *p*-phenylphenacyl ester; the next (like the original mixture of salts) readily yielded the acetate (m.p. and mixed m.p. 111°; cf. Drake & Bronitsky, 1930. Found: C, 75·7; H, 5·5%. Calc. for $C_{16}H_{14}O_3$: C, 75·6; H, 5·5%). The last fractions were rich in formic acid, its presence being confirmed by reducing another portion with magnesium and dilute sulphuric acid, and distilling; the distillate afforded methylene-*bis*-dimedone, m.p. and mixed m.p. 184–188° (cf. Klein & Linser, 1929. Found: C, 69·8; H, 8·0%. Calc. for $C_{17}H_{24}O_4$: C, 69·9; H, 8·2%). A further portion of the salts was steam-distilled from dilute sulphuric acid, and the total acidity and the formic acid content (Auerbach & Zeglin, 1923) determined in the distillate; the formic acid accounted for 50·0% of the acidity.

*Detection of succinic acid.* The bulk of the metabolic solution was reduced to 700–800 ml., then exhaustively extracted with ether, and the ether extract, after drying with anhyd. sodium sulphate, was allowed to evaporate in air in the cold room. A rapid separation of crystals followed. The crystals (1·61 g.), from about 4 g. of total extract, proved to be succinic acid as shown by m.p. and mixed m.p. with an authentic specimen. (Found: C, 40·6; H, 5·2%. Calc. for $C_4H_6O_4$: C, 40·7; H, 5·1%.)

*Detection of lactic acid.* The syrupy residue from which succinic acid had crystallized gave negative results when tested for oxalic, malic, tartaric, and citric acids; it gave no coloration with ferric chloride solution. No appreciable precipitate was produced on treatment first with neutral and then with basic lead acetate. Lead was removed by hydrogen sulphide, the filtrate evaporated below 70° and boiled with water and excess of zinc carbonate. Concentration of the filtered solution yielded zinc lactate, characterized by conversion, via the sodium salt, into *p*-phenylphenacyl lactate, m.p. and mixed m.p. 141° (cf. Drake & Bronitsky, 1930. Found: C, 72·0; H, 5·4%. Calc. for $C_{17}H_{16}O_4$: C, 71·8; H, 5·6%). The total yield corresponded to approximately 2·4 g. lactic acid.

*Detection of glycerol.* The mother liquors from the zinc lactate were freed from a little zinc by sodium carbonate, and treated with benzoyl chloride and sodium hydroxide (Lipp & Miller, 1913), yielding 0·2–0·3 g. tribenzoylglycerol, m.p. and mixed m.p. 71°. (Found: C, 71·1; H, 5·2%. Calc. for $C_{24}H_{20}O_6$: C, 71·3; H, 5·0%.)

*Absence of gluconic acid.* No gluconic acid was found by the application of the method of Birkinshaw & Raistrick (1931) to the residue of the metabolic solution after ether extraction.

### *Metabolic products in absence of serum*

As stated earlier, trypanosomes were also kept in a medium from which serum was absent, and the products of metabolism isolated as described above. The yields were, however, much smaller; the trypanosomes do not survive so well in absence of serum. From the salts of the volatile acids, acetic and formic acids were recognized. Of the volatile acids, 49·7 equivalents % were due to formic acid. Succinic acid and lactic acid were also identified, but the suspected presence of glycerol could not be established.

## DISCUSSION

The main series of experiments on the glucose metabolism of *T. rhodesiense* was carried out with a medium which contained small amounts of serum in order to aid the survival of the parasites, thus increasing the yield of products. Brand (1933) concluded that protein breakdown by suspensions of *T. brucei* was negligible. Reiner *et al.* (1936) also believed that the course of decomposition of glucose by *T. lewisi* and *T. equiperdum* was uninfluenced by the presence of serum. They found, however, that the mechanism of glucose decomposition was very different in the two species of trypanosome. The products obtained in their experiments have all been encountered in the present investigation, in which succinic, pyruvic, lactic, acetic and formic acids, glycerol and ethanol have been identified. Previously, in respiration experiments, the production of small amounts of $CO_2$ was also established.

The formation of large amounts of succinic acid (approx. 40% of the acids extracted) is of great interest. Pasteur (1860) noted that it was formed in small amounts during alcoholic fermentation of sugar by yeast. Ehrlich (1909) suggested that it arose from glutamic acid formed by autolyzed yeast cells, but Kleinzeller (1941) showed that this view was incorrect and discussed the mechanism of the formation of succinic acid from glucose. Wood & Werkman (1940), in their study of the utilization of $CO_2$ by bacteria, noted that inhibition of its use caused a decrease in the amount of succinic acid formed. This fact was considered as evidence that succinic acid might be formed by the union of 1- and 3-carbon compounds, but the condensation of acetic or pyruvic acid was suggested as an alternative method of formation. They drew attention to the possibility that fixation of $CO_2$ with formation of succinic acid may be a general biological phenomenon. Searle & Reiner (1941) showed that *T. lewisi* also assimilated $CO_2$, but such fixation has not so far been demonstrated for *T. rhodesiense*. Krebs (1943) pointed out that most micro-organisms produce $CO_2$ from added substrate, and thus mask a simultaneous uptake. He considered that carboxylation of pyruvic acid is the chief mechanism whereby the 4-carbon chain arises. The observation of Searle & Reiner (1941) that pyruvic acid is not metabolized by *T. lewisi* however suggests some other method of formation.

Lactic and pyruvic acid formation by glycolysis and oxidation is a familiar reaction of many types of cell, and by further oxidation acetic acid may be produced. The formation of formic acid has, in the case of other organisms, been explained by the oxidation of formaldehyde resulting from the reduction of $CO_2$. At present we have no proof that this occurs in the case of *T. rhodesiense*, and aldehyde was never detected in the metabolic solutions. When Reiner & Smythe (1934) investigated the glucose metabolism of *T. equiperdum*, they suggested that the main path of glucose utilization by this organism *in vitro* was as follows:

$$\text{Glucose} \rightarrow \text{glycerol} + \text{pyruvic acid},$$
$$\text{Glycerol} + O_2 \rightarrow \text{pyruvic acid} + 2H_2O.$$

Both pyruvic acid and glycerol have been isolated as metabolic products of *T. rhodesiense* by us. The mode of formation of the ethanol is less clear.

## SUMMARY

1. Products of the glucose metabolism *in vitro* by suspensions of *T. rhodesiense* have been isolated and characterized. They were: succinic, pyruvic, lactic, acetic and formic acids, glycerol, ethanol and carbon dioxide.

2. The possible mode of formation of some of these substances is discussed.

The authors are indebted to Mr J. M. L. Cameron, Department of Chemistry, University of Glasgow, for carrying out the chemical analyses.

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# On the Distribution of Glyoxalase and Glutathione

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(*Received* 13 *June* 1945)

The experiments to be described were directed to determine the presence or absence of glyoxalase and glutathione in such living organisms as have not been previously examined from this point of view. Our results added to those published indicate an exceedingly wide distribution of these cell constituents, especially of the enzyme. On the animal side we have dealt chiefly with invertebrates, which have been but little explored. Dakin & Dudley (1913) stated that glyoxalase is present in the oyster, and Lohmann (1936) found it in the muscle of the octopus. We have been unable to find in the extensive literature on the subject any other similar reference to invertebrates. We have also proved the presence of glyoxalase in several plant species in which its presence has not been previously shown.

## SECTION 1. GLYOXALASE

*Method.* Our method has been that used by Lohmann (1932), Jowett & Quastel (1933), and Platt & Schroeder (1934), namely, the determination of the rate of $CO_2$ evolution, from sodium bicarbonate solution, due to the lactic acid production from methylglyoxal. We employed Barcroft differential manometers provided with standardized flasks. Each flask contained 1·0 ml. of the tissue extract, 0·6 ml. of 0·2 M-sodium bicarbonate, with 2 mg. of glutathione in 0·3 ml. water in a Keilin cup. To one flask, 2 mg. of methylglyoxal were added and the total contents of each flask made up to 3 ml. with distilled water. The apparatus was filled with $N_2 + 5\%$ $CO_2$ gas mixture, the glutathione tipped in and the manometer shaken in a bath at 25°. The stopcocks were left open for 4 min. and then closed.

### *Invertebrates*

In each case the organisms were ground up with sand in a mortar with five times their weight of distilled water. After 30 min. the material was centrifuged, 10 ml. of the supernatant fluid were tied in a short length of cellophan tubing, dialyzed for 24 hr. against distilled water, with frequent changes and then made up to 12 ml. The 1·0 ml. of extract used in the Barcroft apparatus corresponded to 0·166 g. of tissue.

Table 1. *Glyoxalase activity of invertebrates*

| Phylum (and class) | Species | Name | Part used | $\mu$l. $CO_2$ evolved 20 min. | 60 min. |
|---|---|---|---|---|---|
| Coelenterata (Anthozoa) | *Actinia equina* | Anemone | Whole | 129·2 | 327·2 |
| Nematoda | *Ascaris Lumbricoides* | Round worm (pig) | ,, | 405·0 | — |
| Annelida (Polychaeta) | *Aphrodite aculeata* | Sea mouse* | ,, | 84·0 | 240·0 |
| Annelida (Oligochaeta) | *Lumbricus* sp. | Earthworm | ,, | 21·0 | 21·0 |
| Arthropoda (Crustacea) | *Carcinus maenas* | Shore crab | Hepatopancreas | 204·0 | 558·0 |
| ,, | ,, | ,, | Muscle | 82·5 | 244·7 |
| Arthropoda (Insecta) | *Bombyx mori* | Silk worm (larva) | Whole | 96·0 | 261·0 |
| ,, | ,, | Silk worm (pupa) | ,, | 132·0 | 342·0 |
| ,, | *Auriflua* | Caterpillar (larva) | ,, | 45·0 | 99·0 |
| ,, | ,, | Caterpillar (pupa) | ,, | 42·0 | 132·0 |
| ,, | *Locusta* | Locust* | ,, | 321·7 | — |
| ,, | *Athous haemorrhoides* | Wireworm | ,, | 60·0 | 300·0 |
| Mollusca (Gastropoda) | *Helix aspersa* | Garden snail | ,, | 88·0 | 217·2 |
| ,, | *Patella vulgata* | Limpet | ,, | 121·0 | 324·5 |
| Mollusca (Lamellibranchiata) | *Mytilus edulis* | Mussel | ,, | 150·0 | 390·0 |
| ,, | *Paphia* (*Tapes*) sp. | — | ,, | 189·0 | 513·0 |
| ,, | *Chione* (*Venus*) sp. | — | ,, | 93·8 | 239·2 |
| ,, | *Ostrea edulis* | Oyster | ,, | 63·2 | 178·7 |
| Protochordata (Tunicata) | *Ascidiella aspersa* | Sea squirt* | ,, | 39·0 | 102·0 |

* In the case of the sea mouse the cuticle was removed, the test from the sea squirt, and the legs and wings from the locusts.

It will be seen from the results (Table 1) that the concentration of the enzyme (or at least its activity) is considerably less than that recorded in the literature for mammalian organs. It must be remembered, however, that the enzyme is likely to be confined to some special tissue or tissues, so that in the extracts from the whole organism its concentration must be greatly reduced. Again for a similar reason the results do not indicate the relative physiological importance of the enzyme among the different species. Also the temperature employed in the experiments was 25° instead of 37° as used by some authors. Girsavicius, Efendi & Ryzhowa (1936) have shown that the temperature coefficient of glyoxalase in this region is from 1·5 to 1·6. Even for the lowest results given in Table 1 there is evidence that they are due to glyoxalase activity. Whenever the preparation has been completely freed from glutathione by dialysis the evolution of $CO_2$ by the enzyme and methylglyoxal alone is negative or negligible. From the standpoint of present knowledge, at any rate, this would seem to give proof that the specific enzyme is responsible.

### *Plants*

The plants (Table 2) were ground up with sand in a mortar with an equal weight of distilled water, squeezed through linen, and centrifuged. 10 ml. were dialyzed as before. 1 ml. of this was used in the Barcroft apparatus, other details being the same as for the invertebrates.

It will be seen from the above data that the red seaweeds contain a considerable concentration of the enzyme; this also applies to the green varieties we have tested, although here the activity of the

Table 2. *Glyoxalase activity of algae, fungi and conifers*

| Order | Species | Name | Colour | $\mu$l. $CO_2$ evolved 20 min. | 60 min. |
|---|---|---|---|---|---|
| Algae | *Cystoclonium* sp. | Seaweed | Red | 84·0 | 203·0 |
| ,, | *Calliblepharis* sp. | ,, | ,, | 69·0 | 183·0 |
| ,, | *Chondrus crispus* | ,, | ,, | 81·0 | 198·0 |
| ,, | *Rhodymenia palmata* | ,, | ,, | 55·0 | 143·0 |
| ,, | *Ulva latissima* | ,, | Green | 51·0 | 151·0 |
| ,, | *Enteromorpha* sp. | ,, | ,, | 60·5 | 162·2 |
| ,, | *Cladophora* sp. | ,, | ,, | 55·0 | 99·0 |
| ,, | *Fucus spiralis* | ,, | Brown | 36·0 | 87·0 |
| ,, | *Fucus serratus* | ,, | ,, | 18·0 | 39·0 |
| ,, | *Ascophyllum nodosum* | ,, | ,, | 20·5 | 33·4 |
| ,, | *Laminaria digitta* | ,, | ,, | 21·0 | 33·0 |
| ,, | *Laminaria saccharina* | ,, | ,, | 21·0 | 42·0 |
| Fungi | *Penicillium notatum* | Mould | — | 36·0 | 102·0 |
| ,, | *Psalliota campestris* | Mushroom | — | 156·0 | 375·0 |
| Coniferae | *Cypress* sp. | Cypress | — | 27·0 | 39·0 |
| ,, | *Taxus baccata* | Yew | — | 15·0 | 18·0 |
| ,, | *Pinus* sp. | Young pine cones | — | 15·0 | 18·0 |

enzyme is somewhat less. In the brown seaweeds its presence is doubtful except perhaps in *Fucus spiralis*. In the case of the mould studied, although the activity of the enzyme is relatively low, there is no doubt of its presence. In the mushroom its activity is considerable. In those cases where the evolution of $CO_2$ is very low and tends to become very slow after a short period, we feel that the presence of glyoxalase is not proved and our experience leads us to the conclusion that some chemical reaction not due to the enzyme is responsible. When the enzyme is present in adequate amount we have come to believe that this chemical action is so accelerated that the $CO_2$ is evolved during the 4 min. when the manometer stopcocks are open. In the absence of the enzyme results like those seen in the case of the brown seaweeds and the conifers (of which we used young spring growth) are obtained. We believe that glyoxalase is absent from these.

The higher plants (Table 3) were put through a mincer, squeezed through linen, and centrifuged. 10 ml. of the juice were dialyzed as before, and then made up to 20 ml. 1 ml. of this was used in the Barcroft apparatus, other details being the same as in the case of the invertebrates.

Table 3. *Glyoxalase activity of the higher plants*

| Order | Species | Name | Part of plant | µl. $CO_2$ evolved 20 min. | µl. $CO_2$ evolved 60 min. |
|---|---|---|---|---|---|
| Compositae | *Helianthus tuberosus* | Artichoke | Tubers | 46·7 | 118·2 |
| ,, | *Lactuca sativus* | Lettuce | Leaves | 63·2 | 178·7 |
| Cruciferae | *Brassica oleracea* | Cauliflower | Florets | 96·0 | 288·0 |
| ,, | ,, | Cabbage | Leaves | 93·5 | 225·5 |
| ,, | *Nasturtium officinale* | Watercress | ,, | 96·0 | 228·0 |
| ,, | *Raphanus sativus* | Radish | Root | 54·0 | 154·0 |
| ,, | ,, | ,, | Leaves | 68·7 | 167·7 |
| Chenopodiaceae | *Beta vulgaris* | Beetroot | Root | 35·7 | 101·7 |
| Lillaceae | *Allium cepa* | Onion | Bulb | 99·0 | 255·0 |
| ,, | ,, | ,, | Tops | 118·2 | 283·2 |
| Solanaceae | *Solanum tuberosum* | Potato | Mature tubers | 110·0 | 288·7 |
| ,, | ,, | ,, | Leaves | 84·0 | 222·0 |
| Tropaeolaceae | *Tropaeolum majus* | Nasturtium | ,, | 143·0 | 349·0 |
| Urticaceae | *Urtica dioica* | Nettle | ,, | 115·5 | 264·0 |
| Umbelliferae | *Petroselinum sativum* | Parsley | ,, | 255·7 | 649·0 |
| Ranunculaceae | *Delphinium* sp. | Delphinium (blue) | ,, | 48·0 | 138·0 |
| Scrophulariaceae | *Antirrhinum majus* | Antirrhinum (white) | ,, | 30·0 | 78·0 |
| Oleaceae | *Syringa vulgaris* | Lilac (white) | ,, | 21·0 | 30·0 |
| Polemoniaceae | *Phlox* | Phlox (white) | ,, | 39·0 | 102·0 |
| Rosaceae | *Rosa* | Rose (red) | ,, | 33·0 | 39·0 |
| ,, | ,, | ,, | Petals | 15·0 | 21·0 |
| ,, | ,, | Rose (white) | ,, | 21·0 | 51·0 |
| Caryophyllaceae | *Dianthus* sp. | Pink (double white) | ,, | 144·0 | 429·0 |
| Labiatae | *Salvia officinalis* | Sage | Leaves | 22·0 | 27·5 |

To judge from our own observations, and from the results reported in the literature, glyoxalase is widely distributed in the higher plants. Most of the plants which we have examined in fresh condition have contained it. It is perhaps noteworthy that it should be present in green leaves, although long ago Neuberg & Kobel (1926) were the first to show this. It is certainly remarkable that it should be found in the petals of certain flowers, e.g. dianthus (Table 3). Dakin & Dudley (1913) state that they did not find it in young potatoes; it is nevertheless clearly present in mature tubers.

## SECTION II. GLUTATHIONE

*Note on the separation of glutathione.* We have previously shown (Hopkins & Morgan, 1943) that a pure product could be obtained from peas by a relatively simple method. The peas were first ground up in a mortar with 0·1N-$H_2SO_4$ and a little washed sand, the extract centrifuged and the solution half saturated by the addition of solid ammonium sulphate; the filtrate from the precipitate was then brought to 0·5N with respect to $H_2SO_4$. The addition of $Cu_2O$ yielded a completely pure product. It is to be noted, however, that precipitation is delayed in the presence of the ammonium sulphate, and only after standing overnight does a precipitate form. We have since found that the presence of proteins and protein products does not interfere with the precipitation by $Cu_2O$ so that the use of ammonium sulphate may not be necessary. 2 ml. of serum diluted to 50 ml., and 1 g. of proteose preparations in the same volume, gave no trace of precipitate with $Cu_2O$, but when to similar solutions 6 mg. of glutathione were first added precipitation of the cuprous compound was immediate. We have, however, found that the purine bases, but not the pyrimidines, are readily precipitated by $Cu_2O$ from 0·5N-$H_2SO_4$ solutions, and this is important with respect to what follows. Isolation of the tripeptide from the invertebrates presents serious difficulties; the concentrations are in any case relatively low and it has proved difficult in most cases to obtain sufficient of a species at any time, while to accumulate extracts from successive supplies at somewhat long intervals involves risk of removal of the loosely bound glutamic acid from the compound. Moreover, a difficulty presents itself

which is not found in the isolation of the tripeptide from mammalian or from plant tissues, namely, that acid extracts from many invertebrates contain purine bases in considerable quantity, which as stated above are precipitated by $Cu_2O$.

*Invertebrates*

*Earthworms.* From 1700 g. of earthworms we obtained 120 mg. of crystalline glutathione, m.p. 187°. Found: C, 38·63; H, 5·54; N, 13·1; S, 10·19%. Calc.: C, 39·09; H, 5·54; N, 13·68; S, 10·42%. The product, though not quite pure, is nearly so; it had not been recrystallized. *Locusts.* In the case of these insects we were able to take advantage of the circumstance that the purine bases contained in the acid extract are precipitated by $Cu_2O$ immediately, and can then be centrifuged off, while owing to the influence of the ammonium sulphate precipitation of glutathione is delayed for several hours. From 461 g. of whole locusts we obtained 20 mg. of crystalline glutathione (N, 13·4; S, 10·40%).

It is unfortunate that the species of which we could obtain a good supply contained relatively little glutathione and offered special difficulties in attempts at isolation. This is true of the marine mussel, of the garden snail and, when all its soft parts are extracted, of the shore crab. The latter shows some local concentration as the hepatopancreas gives a good nitroprusside reaction, undoubtedly due to glutathione. The extracts of all these species reacted with the enzyme and methylglyoxal in the manometer. When dealing with these species we had not yet observed the interesting circumstance which was afterwards applied to the locusts for the separation of the glutathione from the bases (see above). Owing to the exceedingly large amount of glycogen contained in *Ascaris* we saturated the acid extract from 182 g. with ammonium sulphate and then diluted it with an equal volume of water; after making 0·5N with respect to $H_2SO_4$, $Cu_2O$ gave a small precipitate which was undoubtedly pure glutathione. We obtained later supplies at somewhat long intervals, but in the extract from these the glutathione had decomposed before we attempted isolation. With the anemone, sea-squirt and *Ascaris* we had to rely upon the circumstance that undialyzed aqueous extracts showed a definite glyoxalase activity, which in the current view should constitute indirect proof that glutathione is present in these invertebrates. The amount of extract in the Barcroft flasks corresponded to 2 g. of anemone and sea-squirt tissue, and to 0·5 g. of *Ascaris*. In 1 hr. the amount of $CO_2$ evolved was in the case of the anemone 84, in that of the sea squirt 42, and of *Ascaris* 222 $\mu$l. It is clear that in the two former the concentration of glutathione must be very low.

*Plants*

*Broad bean* (Vicia Faba). From 1400 g., soaked in water for 24 hr. and the skins removed, we obtained 0·38 g. of crystalline glutathione, m.p. 190°. Found: C, 38·93; H, 5·50; N, 13·90; S, 10·33%. *Vegetable marrow* (Cucurbita pepo). We obtained a few mg. of crystalline glutathione, m.p. 188°, from a young vegetable marrow. This was not analyzed, but compared with pure glutathione in the Barcroft apparatus it gave an identical evolution of $CO_2$ with the enzyme and methylglyoxal.

## DISCUSSION

The results of the experiments described add considerably to the knowledge of the distribution of glyoxalase. We have given special attention to invertebrates, which have been very little studied from this point of view. We have found that the enzyme is present in all but one of the sixteen different species from eight different orders examined. It proved to be present in a mould (*Penicillium notatum*) and among algae, in red and green seaweeds, and also in fungi. In addition we have shown its presence in nineteen of the higher plants not previously examined. These results added to the cases described in the literature show how extremely widely distributed is this enzyme in living systems.

The effect must here be considered of the varying concentration of glutathione, the apparently essential concomitant for enzyme activity. In the case of the invertebrates there is no constancy in the relative proportion of the two components present in different species. The earthworm is an extreme but exceptional case of this, in which it is at least doubtful whether the enzyme is present, whereas glutathione is present in considerable amount; but in most species the reverse is the case and the deficiency is in the tripeptide. Glutathione itself is certainly present in the invertebrate order; we have, for instance, isolated it from the earthworm and from the locust, the products proving on analysis to be practically pure. In the case of plants we have not obtained such satisfactory evidence for a relative deficiency in glutathione. We have not attempted to isolate it from the plants in Table 3, and the nitroprusside reaction was obscured owing to the fact that addition of ammonia alone generally produced coloration.

It is quite certain that a deficiency in glutathione may become a limiting factor in the activity of the enzyme. Our measurements of this activity in the various species were made upon extracts which had been dialyzed until the naturally occurring glutathione was removed; an adequate amount of glutathione was then added before the solution was tested in the manometer. The results in Table 1 were thus

obtained. In certain cases, when the extract was not dialyzed and no glutathione was added, the activity proved to be much less than that of dialyzed extract plus glutathione. While the weak activity of the undialyzed extracts might conceivably be due to the presence of some inhibitory material, we found that weak activity goes hand in hand with a weak nitroprusside reaction. Moreover, when to the undialyzed extract 2 mg. of glutathione were added, the activity rose and approximated to that of the dialyzed extract plus glutathione. Thus it seems clear that in the untreated extracts the amount of glutathione present was not sufficient to develop the full activity of the enzyme.

With present knowledge it is, of course, generally assumed that the sole activity of this enzyme is to convert substituted glyoxals into their corresponding hydroxy acids. The absence of satisfactory evidence for the occurrence of such a substrate in the cells and tissues studied has involved disappointment to those interested in the enzyme; this will not be lessened by knowing that the enzyme may exist in certain cells and yet be unable to exert its full activity owing to a deficiency of glutathione.

This failure to gain reliable evidence for any definite function for glyoxalase has now lasted for the thirty years which have elapsed since the discovery of the enzyme by Dakin & Dudley (1913). The realization of this may at the present time be discouraging further work on the subject. We find that some few still hold that in the case of an agent separated from a living system and studied *in vitro* the assumption that it has a function in life partakes of the teleological. It seems more logical to believe that during the processes of development and evolution a functionless agent would have been eliminated, whereas the glyoxalase system remains with its very wide distribution. We feel that our experiments extending the evidence for this should encourage further work in the field. It is possible, though perhaps unlikely, that the enzyme may prove to have a function of a kind not yet recognized, or that its activity has interrelationships with that of other enzymes. We know that this is true in the case of glutathione, especially in the higher animals and plants.

## SUMMARY

1. The results of the experiments described have added considerably to the evidence for the wide distribution of the enzyme glyoxalase in living organisms.

2. While glutathione is also widely distributed, in some cases, especially among the invertebrates, its concentration may be very low and limits the activity of the enzyme actually present.

3. We have isolated glutathione in crystalline form from the earthworm, locust, broad bean and vegetable marrow.

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# The Biological Assay of Vitamin $D_3$. The Effect of the Calcium and Phosphorus Content of the Diet

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(*Received* 23 *May* 1945)

That the content and proportion of calcium and phosphorus have a marked effect on the efficacy of rachitogenic diets used in the biological assay of vitamin D on rats and on the responses of the animals to doses of the vitamin, is well known. Investigations of the effect of the same factors in the assay of vitamin $D_3$ on chicks have been relatively few and in this paper are presented the results of an extended series of investigations of their effect on the growth, tarso-metatarsal distance, and bone-ash of chicks receiving graded doses of vitamin $D_3$ and, in some cases, on serum Ca, P and phosphatase.

In rats it has been shown (Bethke, Kick & Wilder, 1932; Brown, Shohl, Chapman, Rose & Saurwein, 1932; Shohl, 1935, 1936) that growth and production of rickets depend on the level of Ca and P as well

as their ratio in the diet. Rachitogenic diets for vitamin D assay usually have a Ca/P about 0·5/0·12*; diets for optimum growth have Ca : P between 2 and 1.

Such studies on the Ca and P content of the diet in relation to the nutrition of chicks have been concerned with the optimum quantities for growth and egg production for commercial purposes. They show some divergency in results, which is not surprising since other factors in the diet, such as acid-base balance (Titus, McNally & Hillberg, 1933) and nature of the diet, especially the cereal portion (Branion, Stackhouse & Hull, 1938; Loy, DeWitt & Knudsen, 1941; Jones & Elliot, 1943) affect the growth. Usually a ratio of Ca : P between 2 and 1 is considered best for growth and bone formation. Vitamin D requirements diminish as the ratio increases. Jones & Elliot (1943) have suggested that growth of chicks on a suitable diet is a satisfactory criterion for the biological assay of vitamin $D_3$.

The object of the investigations described herein was to determine the optimum conditions for producing maximum differences in response in chicks to graded doses of vitamin $D_3$ and maximum reliability of assays as affected by the calcium and phosphorus content of the diet. The diet was of a cereal type, commonly used in vitamin D studies and previously found (Jones & Elliot, 1943) satisfactory for such investigations.

## METHODS

Sex-linked day-old cockerels (Light Sussex and Rhode Island Reds) were fed on the various diets following the procedure described by Jones & Elliot (1943). After 4 weeks on the vitamin-containing diet and after weighing and radiography, the chicks were killed, and the left tibiae dissected, extracted, dried, and the bone ash determined by the method of the Association of Official Agricultural Chemists (1940). The tarso-metatarsal distances (t.m.t.) were measured on prints from the X-ray negatives. In Exp. 4, the chicks were anaesthetized, and blood removed with a syringe through a direct puncture of the heart. The pooled sample of blood from each group was allowed to clot in a refrigerator overnight, and the serum, poured off next day, was analyzed for calcium, inorganic phosphorus and phosphatase. Calcium was determined by microtitration of precipitated calcium oxalate with 0·01 N-$KMnO_4$ (Hawk & Bergeim, 1942, p. 468), inorganic phosphorus by Kuttner & Cohen's method (Hawk & Bergeim, 1942, p. 461) and phosphatase by Bodansky's method (Hawk & Bergeim, 1942, p. 463; Jones, 1945).

*Basal diet.* The basal diet used had the composition given below and adjustments of calcium and phosphorus content were made by adding the calculated amounts of $CaCO_3$, $Na_2HPO_4$ and bone meal.

* To avoid confusion, all Ca and P contents of the diets are expressed as fractions (e.g. Ca/P, 0·5/0·12) of which the numerator and denominator represent respectively the percentages of Ca and P in the diet.

*Composition of basal diet*

| Material | Percentage |
|---|---|
| Ground yellow corn | 36·34 |
| Middlings | 30·02 |
| Dried skim milk | 9·94 |
| Dried grass meal | 5·82 |
| Soya bean meal | 16·28 |
| Iodized salt (0·02% KI) | 1·06 |
| Charcoal | 0·53 |

*Percentage of calcium and phosphorus in basal diet*

| Exp. | Ca | P |
|---|---|---|
| 1 | 0·32 | 0·78 |
| 2 | 0·29 | 0·78 |
| 3 | 0·29 | 0·77 |
| 4 | 0·30 | 0·73 |

Sufficient basal diet, bone meal, and $CaCO_3$ or $Na_2HPO_4$ were taken to give a total of 99 parts, and 1 of olive oil containing the required amount of vitamin $D_3$ was then added, the whole being mixed in a hobart mixer.

*Vitamin $D_3$ standards.* In Exps. 1, 2 and 4 the standard preparation of vitamin $D_3$ of the British Standards Institution (B.S.I. Specification no. 911, 1940) was used; in Exp. 3, a substandard consisting of a commercial preparation of irradiated 7-dehydrocholesterol which had been found by assay on both rats and chicks to contain 1 million B.S.I. units/g.

## EXPERIMENTAL

The experiments fall into two groups, in one of which (Exps. 1, 3 and 4) the ratio of Ca to P was varied and in the other (Exp. 2) the levels of Ca and P were maintained at what appeared to be the optimum ratio. Exps. 1 and 4 cover values of Ca/P from 1/2 to 2/1. Exp. 3 covers smaller variations from the optimum ratio. Exp. 2 covers the ratio Ca : P = 1 at levels from 0·75 to 3% of both Ca and P.

Previous experience had shown that the growth-promoting and bone-forming effect of vitamin $D_3$ commenced at 10 B.S.I. units/100 g. of the basal diet (Ca/P = 1/1) and was without further effect above 25–30 B.S.I. units/100 g.

In Exp. 1 the levels of vitamin $D_3$ dosage were 0, 10 and 30 units/100 g. of diet; in subsequent experiments the levels were 0, 10 and 22·5 units with additional levels of 15 units in some cases. In Exp. 4 (Ca/P = 1·5/1·5) a range of five levels from 6·7 to 33·75 units was included to check the linear portion of the response curve.

The diets of Exps. 2 and 4 were also fed to rats of 40–50 g. weight newly weaned at 3 weeks of age. Feeding was continued for 4 weeks and gain in weight and bone ash of the femora determined.

The experimental results are summarized in Tables 1 and 2. Table 3 gives relevant statistical data.

The diets of Exps. 2 and 4, when fed to rats, produced no significant differences in weight gains or bone ash. There was normal calcification of the bones on all diets with bone ash averaging 54·7%.

## Table 1. *Experimental data*

(The figures in brackets in the column 'No. of birds' is the number at the commencement of the test. Birds that died were discarded. The high mortality rate on low Ca : P ratios is noteworthy.)

| Exp. no. | Group no. | Percentage of Ca | Percentage of P | Vitamin D (B.S.I. units/100 g. of food) | No. of birds | Mean gain in weight (g.) | Mean t.m.t.* (mm.) | Mean percentage of bone ash | Standard deviations: Gain in weight | Standard deviations: T.m.t.* | Standard deviations: Bone ash | Average food consumption (g.) | Average gain in weight (g./100 g. of food) | Average final weight (g.) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1*a* | 0·32 | 0·78 | 0 | 0 (7) | — | — | — | — | — | — | — | — | — |
| | *b* | | | 10 | 0 (17) | — | — | — | — | — | — | — | — | — |
| | *c* | | | 30 | 12 (17) | 109·0 | 2·875 | — | 30·9 | 0·853 | — | 611 | 17·84 | 180 |
| | 2*a* | 1·0 | 2·0 | 0 | 0 (12) | — | — | — | — | — | — | — | — | — |
| | *b* | | | 10 | 9 (16) | 108·3 | 2·017 | — | 34·2 | 0·473 | — | 566 | 19·16 | 165 |
| | *c* | | | 30 | 15 (17) | 185·0 | 1·200 | — | 38·2 | 0·244 | — | 688 | 26·88 | 245 |
| | 3*a* | 1·0 | 1·0 | 0 | 5 | 141·2 | 4·170 | — | 28·2 | 0·701 | — | 833 | 16·95 | 208 |
| | *b* | | | 10 | 16 (17) | 206·2 | 3·566 | — | 49·8 | 0·787 | — | 747 | 27·63 | 277 |
| | *c* | | | 20 | 16 | 353·1 | 1·316 | — | 53·2 | 0·813 | — | 1017 | 34·72 | 424 |
| | *d* | | | 30 | 15 | 385·8 | 0·867 | — | 24·6 | 0·208 | — | 1069 | 36·09 | 456 |
| | 4*a* | 1·5 | 1·5 | 0 | 5 | 167·2 | 3·880 | — | 18·7 | 1·460 | — | 868 | 19·26 | 242 |
| | *b* | | | 10 | 14 (15) | 251·8 | 2·511 | — | 51·6 | 0·768 | — | 859 | 29·33 | 326 |
| | *c* | | | 30 | 16 | 385·3 | 0·769 | — | 49·1 | 0·191 | — | 1093 | 35·25 | 460 |
| | 5*a* | 2·0 | 1·0 | 0 | 6 | 272·2 | 3·417 | — | 76·6 | 0·605 | — | 1179 | 23·08 | 344 |
| | *b* | | | 10 | 17 | 337·5 | 1·277 | — | 51·2 | 0·573 | — | 1013 | 33·33 | 411 |
| | *c* | | | 30 | 13 (15) | 342·9 | 0·754 | — | 64·5 | 0·161 | — | 1069 | 32·07 | 419 |
| 4 | 1*a* | 1·0 | 2·0 | 0 | 0 (10) | — | — | — | — | — | — | — | — | — |
| | *b* | | | 10 | 9 (18) | 133·3 | 2·589 | 39·14 | 32·6 | 1·047 | 2·10 | 683 | 21·21 | 193 |
| | *c* | | | 22·5 | 16 (17) | 204·3 | 1·200 | 45·75 | 39·9 | 0·330 | 1·95 | 825 | 24·76 | 264 |
| | 2*a* | 1·0 | 1·0 | 0 | 7 | 149·0 | 3·400 | 36·86 | 31·1 | 0·914 | 1·44 | 733 | 20·33 | 212 |
| | *b* | | | 10 | 15 (16) | 226·9 | 2·923 | 37·94 | 67·7 | 0·779 | 1·40 | 893 | 25·42 | 297 |
| | *c* | | | 22·5 | 16 | 344·1 | 0·981 | 43·77 | 54·8 | 0·467 | 2·47 | 1108 | 31·05 | 411 |
| | 3*a* | 1·5 | 1·5 | 0 | 8 | 150·6 | 3·744 | 34·43 | 32·3 | 1·030 | 2·06 | 756 | 19·92 | 222 |
| | *b* | | | 6·7 | 14 (17) | 205·3 | 2·950 | 36·76 | 49·4 | 1·050 | 2·09 | 898 | 22·86 | 277 |
| | *c* | | | 10 | 16 (17) | 200·8 | 3·087 | 36·10 | 68·5 | 1·111 | 2·27 | 788 | 25·46 | 270 |
| | *d* | | | 15 | 17 | 321·5 | 1·759 | 41·67 | 67·0 | 0·924 | 2·65 | 1028 | 31·28 | 389 |
| | *e* | | | 22·5 | 17 | 364·6 | 0·712 | 44·76 | 38·7 | 0·303 | 1·84 | 1092 | 33·38 | 437 |
| | *f* | | | 33·75 | 17 | 360·5 | 0·626 | 45·41 | 57·5 | 0·669 | 1·38 | 1059 | 34·04 | 432 |
| | 4*a* | 2·0 | 1·0 | 0 | 9 (10) | 289·8 | 3·089 | 36·33 | 52·8 | 0·848 | 0·94 | 1106 | 26·21 | 359 |
| | *b* | | | 10 | 18 | 366·0 | 0·858 | 43·31 | 47·3 | 0·367 | 2·75 | 1087 | 33·67 | 438 |
| | *c* | | | 22·5 | 16 (17) | 345·3 | 0·481 | 45·58 | 47·6 | 0·168 | 1·32 | 1066 | 32·38 | 415 |
| 3 | 1*a* | 1·0 | 1·5 | 0 | 2 (7) | 81·0 | 2·725 | 34·31 | 11·3 | 1·025 | 4·24 | 586 | 13·82 | 158 |
| | *b* | | | 10 | 12 (18) | 106·8 | 3·350 | 37·85 | 38·9 | 1·690 | 4·53 | 621 | 17·20 | 189 |
| | *c* | | | 22·5 | 17 | 192·9 | 2·768 | 39·19 | 80·3 | 0·665 | 2·84 | 739 | 26·09 | 271 |
| | 2*a* | 1·25 | 1·5 | 0 | 7 (6) | 102·5 | 3·188 | 36·51 | 21·9 | 1·518 | 4·04 | 735 | 13·94 | 184 |
| | *b* | | | 10 | 15 (17) | 139·1 | 3·023 | 38·94 | 52·3 | 1·122 | 3·35 | 690 | 20·15 | 216 |
| | *c* | | | 22·5 | 16 | 310·8 | 2·053 | 41·54 | 74·7 | 0·609 | 1·71 | 955 | 32·54 | 388 |
| | 3*a* | 1·5 | 1·5 | 0 | 3 (5) | 170·7 | 3·467 | 35·15 | 21·4 | 0·813 | 2·74 | 875 | 19·50 | 241 |
| | *b* | | | 10 | 14 | 174·0 | 3·086 | 38·27 | 42·5 | 0·996 | 2·45 | 813 | 21·41 | 251 |
| | *c* | | | 15 | 14 | 248·9 | 2·396 | 38·43 | 79·9 | 0·828 | 1·91 | 853 | 29·18 | 323 |
| | *d* | | | 22·5 | 14 | 358·7 | 1·314 | 43·50 | 84·7 | 0·757 | 2·08 | 1077 | 33·30 | 434 |
| | 4*a* | 1·5 | 1·25 | 0 | 7 | 190·3 | 3·557 | 35·40 | 33·4 | 0·686 | 2·45 | 854 | 22·28 | 271 |
| | *b* | | | 10 | 14 | 232·3 | 2·836 | 35·84 | 36·4 | 1·064 | 2·32 | 771 | 30·15 | 313 |
| | *c* | | | 22·5 | 16 | 400·8 | 0·728 | 42·98 | 63·6 | 0·370 | 2·07 | 1129 | 35·51 | 480 |
| | 5*a* | 1·5 | 1·0 | 0 | 7 | 225·1 | 3·150 | 33·28 | 33·3 | 0·539 | 1·63 | 907 | 24·82 | 302 |
| | *b* | | | 10 | 15 | 353·3 | 2·080 | 36·96 | 60·7 | 0·854 | 2·33 | 1115 | 31·68 | 431 |
| | *c* | | | 22·5 | 15 | 426·7 | 0·757 | 43·44 | 59·2 | 0·301 | 1·46 | 1230 | 34·69 | 507 |
| 2 | 1*a* | 0·76 | 0·76 | 0 | 9 (10) | 121·0 | 4·511 | 28·64 | 24·3 | 0·786 | 2·93 | 675 | 17·93 | 194 |
| | *b* | | | 10 | 18 (19) | 139·3 | 3·833 | 29·92 | 32·1 | 0·969 | 2·25 | 652 | 21·36 | 212 |
| | *c* | | | 22·5 | 18 | 225·4 | 2·378 | 36·58 | 67·8 | 0·964 | 2·83 | 865 | 26·07 | 297 |
| | 2*a* | 1·0 | 1·0 | 0 | 9 (10) | 134·1 | 3·767 | 31·15 | 33·4 | 0·612 | 2·34 | 704 | 19·05 | 209 |
| | *b* | | | 10 | 16 (19) | 162·4 | 3·219 | 32·91 | 35·6 | 0·785 | 2·23 | 753 | 21·56 | 236 |
| | *c* | | | 22·5 | 17 (18) | 315·7 | 1·385 | 40·83 | 71·1 | 0·920 | 2·83 | 975 | 32·39 | 376 |
| | 3*a* | 1·25 | 1·25 | 0 | 9 | 160·4 | 3·767 | 33·20 | 39·1 | 1·011 | 1·80 | Not taken | — | 229 |
| | *b* | | | 10 | 18 (20) | 163·2 | 2·908 | 35·03 | 54·6 | 1·047 | 2·25 | 685 | 23·84 | 242 |
| | *c* | | | 22·5 | 18 (19) | 344·0 | 0·942 | 43·80 | 60·6 | 0·556 | 2·03 | 979 | 35·13 | 404 |
| | 4*a* | 1·5 | 1·5 | 0 | 10 | 139·7 | 3·420 | 32·57 | 21·0 | 1·103 | 1·95 | 750 | 18·64 | 216 |
| | *b* | | | 10 | 19 | 178·2 | 2·884 | 34·60 | 49·7 | 0·661 | 1·09 | 724 | 24·62 | 256 |
| | *c* | | | 22·5 | 20 | 365·4 | 0·760 | 43·46 | 40·5 | 0·210 | 2·01 | 1002 | 36·49 | 431 |
| | 5*a* | 2·0 | 2·0 | 0 | 10 | 133·3 | 3·130 | 35·57 | 23·2 | 1·307 | 1·99 | 697 | 19·12 | 197 |
| | *b* | | | 10 | 19 | 148·6 | 2·584 | 36·88 | 33·8 | 0·845 | 2·48 | 704 | 21·12 | 224 |
| | *c* | | | 22·5 | 20 | 255·2 | 1·395 | 43·63 | 50·7 | 0·754 | 2·35 | 901 | 28·32 | 329 |
| | 6*a* | 3·0 | 3·0 | 0 | 2 (8) | 89·0 | 2·400 | 40·83 | 1·41 | 0·707 | 1·71 | 565 | 15·76 | 152 |
| | *b* | | | 10 | 11 (14) | 118·2 | 2·091 | 40·91 | 26·3 | 0·480 | 1·95 | 715 | 16·52 | 186 |
| | *c* | | | 22·5 | 18 (19) | 185·8 | 1·419 | 46·22 | 49·6 | 0·384 | 3·14 | 731 | 25·40 | 253 |

* t.m.t.=tarso-metatarsal distances.

Table 2. *Serum calcium, phosphorus, and phosphatase (Exp. 4)*

| Percentage of Ca | Percentage of P | Vitamin $D_3$ (B.S.I. units/100 g. diet) | Serum Ca (mg./100 ml.) | Serum inorganic P (mg./100 ml.) | Serum phosphatase (Bodansky unit/100 ml.) |
|---|---|---|---|---|---|
| 1 | 2 | 0 | — | — | — |
| | | 10 | 11·5 | 10·7 | 127·7 |
| | | 22·5 | 15·9 | 8·5 | 34·6 |
| 1 | 1 | 0 | 9·4 | 5·9 | 183·5 |
| | | 10 | 9·6 | 6·2 | 152·1 |
| | | 22·5 | 8·1 | 7·6 | 53·3 |
| 1·5 | 1·5 | 0 | 7·6 | 9·4 | 176·4 |
| | | 6·7 | 7·3 | 6·2 | 159·4 |
| | | 10 | 7·0 | 6·8 | 185·6 |
| | | 15 | 11·1 | 7·9 | 71·3 |
| | | 22·5 | 11·6 | 6·1 | 33·7 |
| | | 33·8 | 12·3 | 6·8 | 29·1 |
| 2 | 1 | 0 | 7·7 | 5·2 | 167·2 |
| | | 10 | 11·8 | 7·6 | 43·8 |
| | | 22·5 | 12·4 | 14·5 | 26·4 |

(*b*) With adequate dosage of vitamin $D_3$ the differences produced by variations in the Ca and P content of the diet tend to disappear when the Ca/P is greater than 1/1.

(*c*) An amount of 1·5% Ca and 1·5% P in the diet gives optimum increase of response with graded increase of vitamin $D_3$ intake.

(*d*) With a Ca : P ratio of 1, gain in weight increases with increase in dietary level of Ca and P up to 1·5%, after which it decreases. This occurs at all dosage levels of vitamin $D_3$. In the case of t.m.t. responses, a corresponding phenomenon occurs on the highest vitamin $D_3$ dose level, the t.m.t. responses reaching a minimum at 1·5% Ca and 1·5% P and then increasing again. No such reversal occurs in the case of the percentage of bone ash (Exp. 2).

(*e*) Data for efficiency of food utilization (gain in weight/100 g. of food consumed) run parallel with the absolute gain in weight of the chicks. The increased food consumption with increase in vitamin $D_3$ content of the diet is most marked and is accompanied by an increased efficiency of utilization.

(*f*) The dietary Ca does not appear to affect the serum Ca to any great extent under the conditions of Exp. 4, the value being 7·5–9·5 mg./100 ml. of serum in the absence of vitamin $D_3$ and increasing to 11–12 mg. with intake of the vitamin. Inorganic phosphorus does not appear to be affected, but serum phosphatase follows the same course as in other animals, being considerably reduced with increased

Table 3. *Regression data*

| Exp. no. | Group no. | Percentage of Ca | Percentage of P | Gain in weight (g.) $b$ | Gain in weight (g.) $b^2/s^2$ | Tarso-metatarsal distances (mm.) $b$ | Tarso-metatarsal distances (mm.) $b^2/s^2$ | Bone ash (%) $b$ | Bone ash (%) $b^2/s^2$ |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 1·0 | 2·0 | 136·4 | 13·7 | −1·453 | 17·7 | — | — |
| 10 and 30 units | 3 | 1·0 | 1·0 | 283·7 | 51·2 | −4·264 | 52·3 | — | — |
| | 4 | 1·5 | 1·5 | 229·5 | 20·8 | −2·995 | 30·6 | — | — |
| | 5 | 2·0 | 1·0 | 10·8 | 0·04 | −1·044 | 5·5 | — | — |
| 4 | 1 | 1·0 | 2·0 | 150·8 | 16·1 | −2·953 | 19·3 | 14·05 | 49·3 |
| 10 and 22·5 units | 2 | 1·0 | 1·0 | 262·5 | 18·4 | −4·332 | 47·7 | 13·06 | 41·6 |
| | 3 | 1·5 | 1·5 | 336·9 | 32·1 | −4·799 | 32·2 | 17·55 | 72·6 |
| | 4 | 2·0 | 1·0 | −60·3 | 1·6 | −1·097 | 14·2 | 6·62 | 9·1 |
| 3 | 1 | 1·0 | 1·5 | 201·2 | 9·1 | −1·365 | 1·3 | 3·13 | 0·75 |
| 10 and 22·5 units | 2 | 1·25 | 1·5 | 348·0 | 28·8 | −1·967 | 4·8 | 5·27 | 4·0 |
| | 3 | 1·5 | 1·5 | 387·4 | 33·4 | −3·736 | 17·9 | 18·25 | 83·5 |
| | 4 | 1·5 | 1·25 | 325·6 | 36·1 | −4·072 | 27·6 | 13·79 | 39·8 |
| | 5 | 1·5 | 1·0 | 185·8 | 9·6 | −3·359 | 27·3 | 16·40 | 71·0 |
| 2 | 1 | 0·75 | 0·76 | 181·4 | 11·7 | −3·067 | 10·1 | 14·02 | 30·0 |
| 10 and 22·5 units | 2 | 1·0 | 1·0 | 330·3 | 33·8 | −3·902 | 20·7 | 17·05 | 44·4 |
| | 3 | 1·25 | 1·25 | 356·4 | 38·3 | −3·877 | 21·4 | 17·28 | 64·9 |
| | 4 | 1·5 | 1·5 | 379·4 | 70·4 | −4·304 | 78·7 | 17·96 | 121·7 |
| | 5 | 2·0 | 2·0 | 231·9 | 28·7 | −2·589 | 10·5 | 14·68 | 37·0 |
| | 6 | 3·0 | 3·0 | 186·8 | 19·4 | −1·629 | 14·9 | 14·65 | 28·2 |

*Note.* $b$ is the slope of the regression line. $s^2/b^2$ is the major factor influencing the value of the semi-fiducial range of an experiment or assay. (See British Standards Institution Specification 911/1940, App. C, p. 16.) The smaller is this factor, the narrower the fiducial limits or the greater the reliability of the results. The author prefers to use the inverse value $b^2/s^2$ which is a direct index of reliability, i.e. the greater the value, the greater the reliability.

## DISCUSSION

Consideration of the data of Tables 1 and 3 shows that:

(*a*) Gain in weight and t.m.t. were found to vary with the Ca : P ratio (within the range of 0·5–2·0) of the diet, the former rising and the latter falling with increasing ratio. The percentage of ash in the bones did not vary with the Ca : P ratio within the same range. The effect of vitamin $D_3$ on increase in weight diminished with increase in the Ca : P ratio.

intake of vitamin D and showing an **S** type of curve analogous to those for other criteria of response (Table 2). The figures confirm those of Correll & Wise (1938).

(*g*) The regression of gain in weight, t.m.t., and bone ash on log vitamin intake for Ca/P = 1·5/1·5 is statistically linear over the range 10–22·5 B.S.I. units/100 g. of diet. This confirms the findings of Motzok, Hill, Branion & Slinger (1943). The data of the bone ash from Exp. 3, Ca/P = 1·5/1·5, provide the sole anomaly.

(*h*) Table 3 shows that the slope of the regression line and the reliability of assays carried out under these experimental conditions, judged from values of $b^2/s^2$, rises to a maximum at Ca/P = 1·5/1·5 and then falls (Exps. 2 and 4). This is partially confirmed in Exp. 3 in which there is a finer gradation of Ca : P ratios. Covariance analysis reveals a significant variation at the 99·9% level of probability among the diets, which is associated with Ca and P content. The data of the bone ash from Exp. 3 are an exception.

Differences in the slopes of the regression lines are significant in all cases for P = 0·95 in Exps. 1 and 4. In Exp. 3, the following differences are significant: gain in weight among groups 1, 3 and 5; t.m.t. among groups 1, 3 and 5; bone ash among groups 1, 2 and 3. In Exp. 2, the bone ash slopes do not differ significantly, but the slopes for groups 1, 5 and 6 (gain in weight) are significantly less than those for groups 2, 3 and 4, which do not differ significantly among themselves. Also, the slopes for groups 5 and 6 (t.m.t.) are significantly less than those for groups 1–4.

No significant difference is found between the regressions on the same diet in different experiments.

The standard deviations of percentages of bone ash compare favourably with published values, viz. ±2·49 and ±3·63 (Oser (1939) and Motzok *et al.* (1943) respectively).

The present study confirms the suggestion previously made (Jones & Elliot, 1943) that the chick-growth method is a simple, economic and accurate method of biological assay for vitamin $D_3$.

## SUMMARY

1. The effect of variation of the Ca and P content of a standard diet on the responses of chicks to graded doses of vitamin D has been studied. The response criteria utilized were gain in weight, tarso-metatarsal distance and percentage of bone ash of tibiae.

2. The maximum spread of average responses and consequently the steepest slope of the response line and the maximum reliability of assays was obtained with 1·5% Ca and 1·5% P in the diet. Variations of ±20% in these amounts showed no significant change in the slope and reliability.

3. The regression of response on log dose on this diet is linear between 10 and 22·5 B.S.I. units of vitamin $D_3$/100 g. of food.

4. The bone ash criterion of response gives a greater reliability of assays carried out under these experimental conditions than the gain in weight or t.m.t. methods. Both the latter methods are simpler and more economical. The gain in weight method requires no special apparatus and is suggested as very suitable for routine assays.

5. Data for serum Ca, P and phosphatase are also presented. A marked fall in serum phosphatase with increase in vitamin $D_3$ intake is shown.

The author's thanks are due to Miss J. F. Elliot for supervision of the animals and to the Directors of British Colloids Ltd. for permission to publish this work.

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# Biochemical Characterization of Actions of Chemotherapeutic Agents

## 5. LACK OF GROSS DISPLACEMENT OF PANTOTHENATE AND *p*-AMINOBENZOATE FROM MICRO-ORGANISMS, BY PANTOYLTAURINE AND SULPHANILAMIDE

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(*Received* 27 *July* 1945)

The bacteriostatic actions of sulphanilamide and pantoyltaurine have many characters in common, including those associated with their antagonism by compounds which are structurally related to the inhibitors and which are of natural occurrence. In the case of antagonism by *p*-aminobenzoate to the action of sulphanilamide, Woods (1940) and Fildes (1940) concluded that the interaction was by a competitive mechanism. A more quantitative demonstration of this was given by Wyss (1941); he, with later workers, based his analysis of the antagonism on equations which assumed equilibria between the reactants and some component of the micro-organisms. The equilibria implied that (in a sense to be discussed) the amount of free *p*-aminobenzoic acid in a system containing susceptible bacteria and a limited amount of the acid would increase on addition of sulphanilamide; Fildes (1940) and Harris & Kohn (1941) anticipated such 'displacement'. Similar characters have been shown, and mechanisms assumed, for the antagonism by pantothenate to the antibacterial action of pantoyltaurine (McIlwain, 1942, 1944). In neither case has the 'displacement' been directly observed, and the biological conditions under which it might occur, or the quantities of substances which it might involve, have not been specified. Although Green (1940) indicated that no considerable liberation of sulphonamide antagonists from *Brucella abortus* was caused by sulphanilamide, a displacement of *p*-aminobenzoic acid adsorbed on charcoal or a blocking of the adsorption, by sulphonamides, have been considered as models of the actions of the drugs by Hartmann & Druey (1943) and Eyster (1943).

The possible displacement of *p*-aminobenzoate and pantothenate from microbial cells has now been examined. The method adopted in the case of pantothenate permitted observation of the release, from a small quantity of cells, of about 1/1000 of their pantothenate content. As it was not found possible by Feinstone, Williams & Florestano (1942) to correlate the absorption of sulphonamides by bacteria with their biological action (as was the case with arsenicals: Hawking, 1937, 1938), any observation of displacement would have additional significance as affording a possible connexion between the changed biological behaviour of susceptible organisms in the presence of the inhibitors and changes in their cell substance.

### EXPERIMENTAL

*Preparation of the streptococci.* The Richards (group A) *Streptococcus haemolyticus* and a group G organism, maintained as previously described (McIlwain, 1944), were prepared for the present experiments by growth in a medium, based on a casein hydrolysate (McIlwain & Hughes, 1944), under conditions which yielded cells of varying pantothenate-content. Pantothenate-poor cells were obtained from media initially containing $10^{-6}$M-pantothenate, in which growth was allowed to proceed for 30–42 hr., with progressive neutralizing of the acid formed by glycolysis (McIlwain & Hughes, 1944). After such growth, the pantothenate concentration of the medium was $<10^{-8}$M; these bacteria were examined without washing. Pantothenate-rich cells were produced by growth for 17–22 hr. in media whose initial pantothenate concentration of $10^{-5}$M did not fall below $10^{-6}$M when the organisms were harvested. This was done by centrifuging, and organisms from 100 ml. of medium were washed twice with 10 ml. of 0·5% NaCl, which did not remove all the pantothenate loosely associated with them (see below); they were used in the following experiments within 2 hr. of harvesting.

*Effect of reagents on streptococcal pantothenate.* Bacterial suspensions were divided into portions containing the equivalent of 15–60 mg. dry wt. of cells in M/18 phosphate (pH 7·6) and having a final volume of 2–5 ml. which contained the reagents whose effects were being studied (Tables 1 and 2). In the three cases examined, photometric measurement of the turbidity of portions of the suspension taken before and after such experiments showed no detectable differences; with the apparatus employed, this implied that any difference was $<1·5\%$. After exposure the suspensions were adjusted to 10 ml., the fluids separated by centrifuging, sterilized by filtration, and their pantothenate content determined. The cells, usually without washing, were digested to release pantothenate (McIlwain & Hughes, 1944), which in all cases was determined by growth of *Proteus morganii*, measured photoelectrically (McIlwain & Hughes, 1945). In a few experiments, using high pantoyltaurine concentrations and desoxycholate, cells were washed before digestion in case these reagents affected the liberation of pantothenate, but such effects were not found; pantothenate in the washings was negligible.

*Effect of sulphanilamide on the liberation of its antagonists from streptococci.* The Richards Streptococcus was grown for 17–20 hr. in the media described above, initially containing $10^{-6}$M-pantothenate. Exposure to buffer and saline solutions, with and without sulphanilamide, was carried out

on batches of 1 g. (wet wt.) of the washed cells by the method described below; no release of sulphanilamide antagonists was detected. After exposure, antagonists were liberated by autolysis with ammoniacal solutions, as described by Stamp (1939). For further experiments yeast was chosen as a material which is accessible in larger quantities and whose sulphonamide antagonists have been much studied.

*Effect of sulphanilamide on the liberation of its antagonists from yeast.* Commercial yeasts were used; their growth was retarded by 0·02 M-sulphanilamide. A batch of bakers' and a batch of brewers' yeast were each suspended in 10 vol. of 0·5% NaCl, separated by centrifuging, and the washing repeated. Sulphanilamide (2 g.) and water (5 ml.) were added to 20 g. portions of the moist cells and the mixtures rubbed occasionally during 4 hr. at 37°. Similar portions of the yeasts were treated in the same way, but without addition of sulphanilamide. All were centrifuged, and the supernatants without sulphanilamide were saturated with the drug. All were kept at 0° overnight to remove the bulk of sulphanilamide. This arrangement of control experiments was chosen to avoid unequal losses (e.g. by adsorption or in crystals) from the different solutions, or unequal inhibition by them in testing. The recovery of added *p*-aminobenzoate from such a process was examined, and >90% was found to be recovered.

The total antagonists of the yeasts were determined by autolysis of portions for 12 hr. under Stamp's (1939) conditions; all solutions were assayed for antagonists by their effects on growth of the Richards Streptococcus in a peptone medium (McIlwain, Fildes, Gladstone & Knight, 1939; Woods, 1940) containing $3·3 \times 10^{-4}$ M-sulphanilamide. Growth from a series of dilutions of the extracts was compared with that produced by a reference series of dilutions of *p*-aminobenzoate, and the results of assays expressed as the quantities of *p*-aminobenzoate which would be required to produce the observed antagonism. Interaction between the material from yeast or streptococci, and sulphanilamide, was found to be competitive.

## RESULTS

### *Behaviour of the pantothenate of streptococcal cells*

*Pantothenate content.* Experiments attempting to displace pantothenate were first carried out with organisms grown in media whose initial pantothenate concentration ($2 \times 10^{-6}$ M) approximated to that of mammalian tissue fluids. The pantothenate content of the bacteria (Table 1) was about 30 mμmol./g. dry weight. These values are low in comparison with those found by Thompson (1942) in other organisms, though the method of extraction was similar to that used by Thompson and its applicability to the present streptococci has been confirmed (McIlwain & Hughes, 1944). It was then found that streptococci richer in pantothenate could be obtained by growth for shorter periods; these differences were correlated with the pantothenate content of growth media at the time of harvesting the cells. Although the medium first used contained about 50 times the quantity of pantothenate of the harvested cells, their inactivation of pantothenate (McIlwain & Hughes, 1944) had left the medium containing $< 10^{-8}$ M-pantothenate at the time when the bacteria were collected. With shorter growth periods and higher initial pantothenate concentrations, cells containing up to 700 mμmol./g. dry weight were obtained (Table 2). Cells containing

Table 1. *Effect of pantoyltaurine on the pantothenate of pantothenate-poor streptococci*

Organism: Group G Streptococcus. Exposure to reagents for 4 hr. at 37° was in 2–5 ml. of phosphate buffer (pH 7·6) unless otherwise described (column 2). The casein hydrolysate was that of McIlwain & Hughes (1944).

| Batch of organisms; dry wt. (mg.) | Reagents and conditions of exposure | Pantothenate: In solution after exposure — Total (mμmol.) | In solution after exposure — mμmol./g. dry wt. of organisms | On later extraction of exposed organism — Total (mμmol.) | On later extraction of exposed organism — mμmol./g. dry wt. of organisms | Total, from solution and cells (mμmol./g. dry wt. of organisms) |
|---|---|---|---|---|---|---|
| I; 44 | Buffer only | 0·2 | 4·5 | 1·0 | 23 | 27·5 |
| ,, | Pantoyltaurine, 200 μmol. | 0·2 | 4·5 | 0·95 | 22 | 26·5 |
| ,, | Pantoyltaurine, 20 μmol. | 0·3 | 7 | — | — | — |
| ,, | Desoxycholate, 1 mg. | 0·25 | 6 | — | — | — |
| II; 43 | Buffer only | 0·3 | 7 | — | — | — |
| ,, | Pantoyltaurine, 100 μmol. ± urea, 2 mmol. | 0·25 | 6 | — | — | — |
| ,, | Urea, 2 mmol. | 0·35 | 8 | — | — | — |
| IV; 44 | (Not exposed) | — | — | 1·25 | 28·5 | 28·5 |
| ,, | Casein hydrolysate, 10 mg.; glucose, 0·25 mmol. | 0·25 | 6 | 0·5 | 11 | 17 |
| ,, | Casein hydrolysate, 10 mg.; glucose, 0·25 mmol. and pantothenate, 10 mμmol. | 0·88 | — | 0·5 | 11 | — |
| ,, | Casein hydrolysate, 10 mg.; glucose, 0·25 mmol. and pantothenate, 10 mμmol. and pantoyltaurine, 100 μmol. | 8·8 | — | 0·75 | 17 | — |
| V; 48 | At pH 9·0 | 0·13 | 2·5 | — | — | — |
| ,, | At pH 7·6 ± pantoyltaurine, 100 μmol. | 0·13 | 2·5 | 1·20 | 25 | 27·5 |
| ,, | At pH 5 | 1·02 | 21 | 0·45 | 9 | 30 |

the lower quantities of pantothenate remained viable, and lesser quantities were not found in freshly grown cells prepared with less pantothenate.

*Stability of pantothenate in pantothenate-poor streptococci.* Table 1 shows relatively little pantothenate to be liberated into solution from washed organisms of 'pantothenate-poor' type, under a variety of conditions. Pantoyltaurine was used in concentrations up to 50,000 times that of the total pantothenate; ratios of 100 and 20 inhibit respectively growth and pantothenate inactivation by the bacteria. Little inactivation of pantothenate was observed under the conditions of exposure of batch I, Table 1, and the presence of pantoyltaurine would tend to protect any of the acid which it liberated to solution. Urea and desoxycholate did not increase the pantothenate extracted, either with or without pantoyltaurine. Together with previous results (Cheldelin, Eppright, Snell & Guirard, 1942; Thompson, 1942; McIlwain & Hughes, 1944) the present observations suggest the pantothenate to be chemically linked in the bacteria. Liberation by autolysis has been found maximal at acid pH; at pH 5, much pantothenate was freed, but pantoyltaurine did not increase that amount. Some of the pantothenate of streptococci disappeared in mixtures which permitted glycolysis (batch IV, Table 1) and pantoyltaurine decreased the quantity of pantothenate which disappeared but not the amount of pantothenate left in the cells.

*Stability of pantothenate of pantothenate-rich streptococci.* A large part of the pantothenate of such organisms, though remaining associated with them after washing at room temperature, was released into saline solutions at 37° (Table 2); the quantity remaining approximated to that shown by the pantothenate-poor preparations. The most sensitive indication of possible displacement of pantothenate with the present organisms was thus the amount remaining in their cells and liberated by later digestion. Large excesses of pantoyltaurine had little effect on these amounts (Table 2). Some pantothenate disappeared during exposure, but was protected by pantoyltaurine; phenomena of this type occur during glycolysis (McIlwain & Hughes, 1944).

*Table 2. Effect of pantoyltaurine on the pantothenate of pantothenate-rich streptococci*

| Strain and batch of organisms; dry wt. (mg.) | Pantoyltaurine (μmol.) in 3 ml. | Pantothenate: In solution after exposure — Total (mμmol.) | In solution after exposure — mμmol./g. dry wt. of organisms | On later extraction of exposed organisms — Total (mμmol.) | On later extraction of exposed organisms — mμmol./g. dry wt. of organisms | Total, from solution and cells (mμmol./g. dry wt. of organisms) |
|---|---|---|---|---|---|---|
| Group G, I; 27 | (Not exposed) | — | — | 4·6 | 170 | 170 |
| ,, | 0 | 2·3 | 85 | 0·44 | 16 | 100 |
| ,, | 50 | 4·5 | 165 | 0·43 | 16 | 180 |
| Group G, II; 15·2 | 0 | 6·6 | 435 | 0·37 | 24 | 460 |
| ,, | 50 | 10·8 | 710 | 0·35 | 23 | 730 |
| Group A (Richards); 4·7 | (Not exposed) | — | — | 3·38 | 724 | 724 |
| ,, | 0 | 2·7 | 580 | 0·23 | 49 | 629 |
| ,, | 25 | 3·0 | 640 | 0·22 | 47 | 685 |

mum quantities. Results in which extractions with salt solutions are compared with extractions by salt solutions containing sulphanilamide are given in Table 3. The sulphanilamide was allowed to act for

*Table 3. Liberation of sulphanilamide antagonists from streptococci and yeasts*

| Organism | Antagonists, as *p*-aminobenzoate (mμmol./g. dry wt. of organism) extracted by: Saline | Saline saturated with sulphanilamide at 37° | Autolysis |
|---|---|---|---|
| *Streptococcus haemolyticus* (Richards strain) | <8 | <8 | 45 |
| Yeast (bakers') | 5·5 | 5·5 | 51 |
| Yeast (brewers') | 5·0 | 4·0 | — |

*Behaviour of sulphonamide antagonists of streptococci and yeasts*

Conditions of extraction of sulphonamide antagonists from micro-organisms have not been investigated in the present study as fully as those of pantothenate, for the antagonists in streptococci (Stamp, 1939) and yeast (Woods, 1940) have already been examined with a view to obtaining them in maximum quantities. Results in which extractions with salt solutions are compared with extractions by salt solutions containing sulphanilamide are given in Table 3. The sulphanilamide was allowed to act for a time longer than the latent period in its growth inhibition of sensitive organisms, and in concentrations 100,000 times those of the '*p*-aminobenzoate' which appeared in solution. The antagonism shown by extracted material was similar to that shown by *p*-aminobenzoate in its competitive nature, and previous investigations have shown the bulk of such material from yeast to be *p*-aminobenzoate; it was not further characterized. The quantity of antagonists extracted was small and not appreciably altered by the presence of sulphanilamide; it was a small fraction of that obtainable by autolysis.

## DISCUSSION

The foregoing experiments have demonstrated that there is not a 'displacement' by pantoyltaurine of the bulk of the pantothenate in streptococci, or of the bulk of substances behaving as *p*-aminobenzoate in streptococci and yeast, by sulphanilamide. This contrasts sharply with the effects to be anticipated from certain simple interpretations of the equilibria postulated to explain quantitative characters of the action and antagonism of the two inhibitors. If, however, the existence of equilibria during the functioning of pantoyltaurine and sulphanilamide be accepted, a divergence from the displacement which might be considered the immediate consequence of this hypothesis can be traced, by the following steps, to a definite feature in the action of the inhibitors.

(1) The divergence might be due to the bulk of the pantothenate or *p*-aminobenzoate of the organisms not functioning in the systems through which the inhibitors act. For this to be the case, $>80\%$ of the *p*-aminobenzoate of streptococci, $>95\%$ of the *p*-aminobenzoate of yeast, and $>95\%$ of the pantothenate of streptococci must belong to systems in which the inhibitors do not compete. The experiments with pantoyltaurine included some with streptococci containing minimal quantities of pantothenate, which might be supposed to be present in the most necessary systems, and with which very small quantities of pantoyltaurine interfere in growth. Against the view that pantothenate serves a multiplicity of essential functions, of which that sensitive to pantoyltaurine concerns only a small portion of the organisms' pantothenate, is the observation (McIlwain & Hughes, 1944) that a process of pantothenate metabolism, correlated with the action of pantoyltaurine on growth, is capable of a pantothenate turnover per hour some 50 times greater than that of the quantity of pantothenate in these cells, and is highly sensitive to pantoyltaurine. At least in this case, therefore, explanation (1) (with a reservation to be expressed later) appears to be inadequate; less direct evidence can be adduced to support a similar view concerning the *p*-aminobenzoate of streptococci.

(2) A further possibility is that in the present experiments the inhibitor may not reach the 'site of action', e.g. because of selective permeability. Pantoyltaurine, however, acts on a metabolic process of the organisms which is connected with its action on their growth, within 2 min. of its addition to suspensions identical with those of the present experiments (McIlwain & Hughes, 1944); and, indeed, one of these experiments (batch IV, Table 1) shows the process in operation. Sulphanilamide has been found to penetrate streptococcal cells (Feinstone *et al.* 1942) and its action on growth is manifested within the period used in the present experiments.

(3) It is also possible that the *p*-aminobenzoate and pantothenate, though displaced from essential sites by the inhibitor, might remain otherwise attached to the organisms. In this case the substances would not appear in solution, but their functioning in non-proliferating cells would be disturbed by the inhibitor. Now although much evidence connects that functioning of pantothenate which is inhibited by pantoyltaurine with glycolysis (McIlwain & Hughes, 1944, 1945), non-proliferating streptococci continue their normal reaction with glucose in the presence of high concentrations of pantoyltaurine (McIlwain, 1944). Again, the many attempts which have been made to detect changes in major metabolic activities (such as $O_2$ uptake or $CO_2$ output) following addition of sulphonamides to non-proliferating suspensions of organisms whose growth is susceptible to sulphonamides, have largely failed to give results commensurate with the actions of the drugs on growth. Such negative evidence is, however, a less satisfactory basis for argument than the observation that metabolic defects have been demonstrated in cases in which some growth of the organisms in the presence of the drugs has been allowed (Kohn & Harris, 1941; Sevag & Shelburne, 1942; Henry, 1943).

(4) It is therefore suggested that, although *p*-aminobenzoate and pantothenate function in resting bacteria, these activities, when once the substances are incorporated in the organisms, are not influenced by sulphanilamide and pantoyltaurine, but that the reactions in which the inhibitors act are ones which result in the assimilation, or 'binding' in functioning form, of *p*-aminobenzoate and pantothenate. Evidence that such attachment is by chemical linkages is given by the stability of these substances in bacteria, and by the nature of the processes necessary to extract them. This suggestion agrees with all the foregoing evidence and also with the following points:

(*a*) *The existence of a latent period in the actions of pantoyltaurine and sulphanilamide on growth.* If only assimilation to give the functioning forms of *p*-aminobenzoate and pantothenate is affected, these 'bound' compounds will be available for daughter cells and will continue to perform their normal activities; but their quantity per cell will be reduced at each division which occurs in presence of the inhibitors. Effects are thus to be expected after a period of 'dilution' by growth, the period (which is found to be altered by many circumstances) depending on the adequacy of the original quantities of bound substance. This is consistent with Kohn & Harris's (1941) finding that the later effects of sulphonamides were not increased by prior exposure

of non-proliferating bacteria to the substances. This argument would apply both in the case of *p*-aminobenzoate, which is synthesized by many susceptible bacteria, and in the case of pantothenate, which is not; the production of compounds related to *p*-aminobenzoate can continue in the presence of sulphanilamide (Fox, 1942).

(*b*) *The bacteriostatic, as distinct from bactericidal, effect of the two inhibitors.* If the action of pantoyltaurine is to prevent assimilation of pantothenate, the organisms would be expected, when their growth is inhibited in the presence of both pantothenate and pantoyltaurine, to be in circumstances no more deleterious than those occasioned by absence of pantothenate. This results (McIlwain, 1944), with an otherwise adequate medium, in a gradual cessation of growth, but not in the rapid death produced by bactericidal agents. Although the extent to which streptococci might suffer from lack of *p*-aminobenzoate is unknown it would be unusual for a micro-organism to be killed rapidly by lack of a growth-promoting substance. Non-proliferating bacterial suspensions can show slow rates of 'natural' death (Wilson, 1922; Lodge & Hinshelwood, 1939), which may explain some apparent exceptions (e.g. Colebrook & Cawston, 1945) to the general observation that the sulphonamides are bacteriostatic.

(*c*) *Multiple antagonism to sulphonamide action.* Binding and functioning are here presented as two distinct and successive reactions of a possible series involving *p*-aminobenzoate, the first open to competition with sulphanilamide but the later ones not. Binding of *p*-aminobenzoate then becomes the 'primary reaction' of Harris & Kohn (1941) and bound *p*-aminobenzoate is to be looked upon as a product whose functioning can be partly aided or replaced by secondary antagonists, such as methionine and unknown compounds in peptone. The antagonism shown by these substances, as is necessary to the present scheme, has been found to differ considerably from that shown by *p*-aminobenzoate. Bound pantothenate, similarly, becomes the previously suggested (McIlwain & Hughes, 1945) product of pantothenate metabolism, normally formed in excess of the organism's minimal needs, and already believed to be part of the bacterial cell.

This conclusion respecting *p*-aminobenzoate unites two views of its functioning in susceptible micro-organisms, which have been regarded (Henry, 1943) as mutually incompatible, namely, that it is both a substrate and plays a catalytic role. The first activity is now regarded as occurring primarily in growing cells and to be susceptible to inhibition by sulphonamides; the second activity is regarded as occurring independently of proliferation and to be insusceptible to sulphonamides. The dual aspects of the behaviour of *p*-aminobenzoate which have been separated here, perhaps artificially, make the proposition (1), above, true in one respect and untrue in another, according to whether or not growth is included in the functioning of bacteria; (2) is also ambiguous, as that activity of *p*-aminobenzoate which is inhibited by sulphanilamide is regarded as not operating in non-proliferating cells. Both propositions require the further specification, of a biological nature, which is provided by (3) and (4). An action of pantoyltaurine on the pantothenate of growing, as distinct from non-proliferating, cells has been demonstrated and will be reported later.

## SUMMARY

1. Two strains of $\beta$-haemolytic streptococci, which needed added pantothenate for growth, normally contained 15–50m$\mu$mol. pantothenate/g. dry matter, firmly bound to the bacterial substance. Larger quantities were more loosely associated with the organisms after their harvesting from pantothenate-rich media.

2. The firmly bound pantothenate was liberated on autolysis or enzymic digestion, but not by various reagents which included high concentrations of pantoyltaurine. The loosely attached pantothenate was released into saline solutions at 37°; pantoyltaurine did not decrease the quantity remaining with the organisms.

3. Sulphanilamide did not increase the small quantity of substances antagonizing sulphanilamide bacteriostasis, which were obtainable from streptococci and yeasts by saline solutions.

4. It is concluded, from this and previous evidence, that pantoyltaurine and sulphanilamide act as bacteriostatic agents by preventing the binding, in functioning form, of pantothenate and *p*-aminobenzoate respectively, by susceptible bacteria.

I am indebted to Mr D. E. Hughes for assistance during these investigations.

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# Isolation from Liver of New Factors Essential for the Growth of *Lactobacillus helveticus* and *Streptococcus lactis* R.

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(*Received* 13 *June* 1945)

Snell & Peterson (1940), Stokstad (1941), Mitchell, Snell & Williams (1941), and O'Dell & Hogan (1943) have described the isolation, from various sources, of substances which stimulate the growth of *Lactobacillus helveticus* (*casei* ε) and *Streptococcus lactis* (*faecalis*) R. All of these workers used adsorption on norite and elution with ammoniacal solutions as an essential step in the process of isolation. The factor thus isolated by Mitchell *et al.* (1941) from spinach leaves was named folic acid.

Attempts have been made in the present work to prepare by similar methods a concentrate of folic acid, but using as starting material a precipitate obtained by the addition of ethanol to a concentration of 70% (v/v) during the manufacture of a commercial liver extract ('Plexan', made by Glaxo Laboratories Ltd.) by the method of Laland & Klem (1936). This precipitate was dissolved in water and the solution treated with norite. The adsorbate was eluted by ethanolic ammonia, following the procedure of Snell & Peterson (1940). The resulting eluate stimulated the growth of *L. helveticus* when added to the medium of Landy & Dicken (1942) used for testing for folic acid.

The eluate was next adsorbed on Decalso (an ion-exchange adsorbent made by the Permutit Co. Ltd.), and the adsorbate eluted with hot 10% aqueous sodium chloride solution. Both eluate and filtrate stimulated growth of the test organism. It was also found that at least part of the active material was present in the precipitate formed when silver nitrate was added to the eluate at pH 7. By combining these three methods, there was prepared a concentrate of which 0·5 ml. gave good growth with *L. helveticus*; this concentrate contained under 100 μg. total solids/ml.

Attempts to dissolve the active fraction in chloroform, preparatory to attempting partition chromatography (Martin & Synge, 1941), led to the discovery that most of the activity was extracted by chloroform from both acid (pH 3) and alkaline (pH 11) solutions, although folic acid is said to be insoluble in chloroform. Both the chloroform-soluble and chloroform-insoluble fractions were capable of stimulating the growth of *L. helveticus* and *S. lactis*, and the responsible factors are therefore provisionally designated factor HL 1 and factor HL 2 respectively. The latter may be identical with folic acid.

Extraction of a more refined liver extract ('Examen', made by Glaxo Laboratories Ltd.) by chloroform also gave an active fraction; this, on treatment with Decalso, gave an active filtrate and an eluate that was only slightly active. Similarly, when Examen was run through a column of Decalso and the filtrate and the hot 10% sodium chloride eluate were extracted with chloroform, the extract from the filtrate markedly stimulated the growth of *L. helveticus*, whilst the extract from the eluate gave only partial stimulation. The chloroform-soluble factors present in the Decalso eluate and filtrate are designated factors HL 3 and HL 4 respectively. When the two fractions were combined, growth equal to that given by the chloroform extract from Examen was obtained. It is possible that factor HL 1 is a mixture of factors HL 3 and HL 4.

With one exception, factors HL 1, HL 3 and HL 4 were not affected by acetylation, benzoylation, treatment with nitrous acid or boiling with water, dilute acid or dilute alkali. The activity of HL 4 in the growth of *L. helveticus*, however, was largely destroyed by nitrous acid, but not the activity for *S. lactis*. Its synergistic action with factor HL 3 towards either organism was not decreased.

## EXPERIMENTAL

### *Microbiological procedure*

The organisms used in this investigation were *Lactobacillus helveticus* (*casei* $\epsilon$) strain no. 7469 and *Streptococcus lactis* (*faecalis*) Rogers, which were obtained from the American Type Culture Collection, Georgetown University, Washington, D.C., and *Streptococcus lactis* (National Collection of Type Cultures 8043) obtained from The Lister Institute of Preventive Medicine.

*Preparation of inoculum.* A transfer was made from an agar stab culture to a tube of the Snell & Strong riboflavin test medium (cf. Barton-Wright & Booth, 1943), to which had been added 0·25$\mu$g. riboflavin/10 ml. medium. The time of incubation was 16–18 hr., at 37° for *L. helveticus* and at 30° for *S. lactis* R. After incubation the cultures were centrifuged aseptically, the supernatant medium poured off and 10 ml. of sterile 0·9% saline solution added, followed by aseptic centrifugation. The supernatant saline solution was poured off and the cells were suspended in 10 ml. of 0·9% saline. 1 ml. of this suspension was then added to 100 ml. of sterile saline solution and this diluted suspension was used for inoculation.

The basal medium employed was a slight modification of that described by Landy & Dicken (1942):

| Component | Wt./ml. of basal medium |
|---|---|
| Acid-hydrolyzed casein | 5·0 mg. |
| *dl*-Tryptophan | 0·1 mg. |
| *l*-Cystine | 0·1 mg. |
| Glucose | 10·0 mg. |
| Sodium acetate | 6·0 mg. |
| *d*-Calcium pantothenate | 0·1$\mu$g. |
| Pyridoxin | 0·1$\mu$g. |
| Riboflavin | 0·1$\mu$g. |
| Nicotinic acid | 0·1$\mu$g. |
| *p*-Aminobenzoic acid | 0·1$\mu$g. |
| Biotin | 0·0002$\mu$g. |
| Adenine | 0·01 mg. |
| Guanine | 0·01 mg. |
| Uracil | 0·01 mg. |
| Xanthine | 0·01 mg. |
| Sodium chloride | 5·0 mg. |
| Inorganic salts: solution A and solution B (see below) | 0·5 ml. of each/100 ml. of completed medium |

*Estimation of amount of growth.* The fermentations were carried out in ordinary bacteriological or chemical tubes (18 × 150 mm.), the acid produced being titrated against 0·1N-NaOH with *L. helveticus* and 0·05N-NaOH with *S. lactis* R (Barton-Wright & Booth, 1943). It is necessary to use the lower concentration of standard NaOH for *S. lactis* R, as this organism produces less acid than *L. helveticus*. When the buffer in the medium (sodium acetate) and the glucose are increased to 2%, the titres will be more than doubled. Cultures inoculated with *L. helveticus* were incubated at 37° and those inoculated with *S. lactis* R at 30°, each for 72 hr.

The acid-hydrolyzed casein was prepared from commercial casein and purified by treatment with lead acetate and adsorption on charcoal (10%). *Inorganic salt solution A* was composed of 25 g. $K_2HPO_4$, 25 g. $KH_2PO_4$ in 250 ml. of water. *Inorganic salt solution B* was modified to contain 10 g. $MgSO_4.7H_2O$, 0·5 g. $MnSO_4.4H_2O$, 0·1 g. $FeCl_3$ in 250 ml. of water; five drops of conc. HCl were added to this solution.

### *Separation of active fractions*

The 70% (v/v) ethanol-insoluble precipitate from Plexan was fractionated by adsorption on charcoal and elution with aqueous ethanolic ammonia (Snell & Peterson, 1940), precipitation with silver nitrate solution and adsorption on Decalso. The details of the method are recorded in Fig. 1. Solution A, the charcoal eluate, contained 2·9 mg. total solids/ml., and stimulated the growth of *L. helveticus* when added to the folic acid test medium of Landy & Dicken (1942). Solution B, the filtrate after adsorption by Decalso, had only slight growth-promoting activity, whereas solution C, the Decalso eluate, containing only 0·1 mg. total solids/ml., was very potent.

Solution C was further fractionated by extraction with chloroform after adjusting one part to pH 3 and another to pH 11 (details in Fig. 2). The materials obtained from both chloroform extracts were more active than the residual aqueous solutions. This was a surprising observation since folic acid and related factors have been stated to be insoluble in chloroform (Mitchell *et al.* 1941; O'Dell & Hogan, 1943). Further extraction with chloroform failed to remove any more active material. Since the chloroform-soluble and chloroform-insoluble factors stimulated the growth of *L. helveticus* and *S. lactis*, they are provisionally designated factor HL 1 and factor HL 2 respectively; the latter may be identical with folic acid.

Typical results, indicating the relative activities of the different fractions in stimulating the growth of *L. helveticus*, are given in Table 1.

In view of the separation of a chloroform-soluble growth factor from the 70% ethanol precipitate, it was decided to extract a more refined liver extract (Examen) with chloroform, with the object of obtaining a larger quantity of this factor. The chloroform extract thus obtained was treated with Decalso. The reverse procedure, that is, adsorption of Examen on Decalso followed by chloroform extraction, was also tried. Details of the fractionation are given in Fig. 3, and the relative activities of the fractions in stimulating the growth of *L. helveticus* in Table 2.

The chloroform extract proved to be extremely potent and, on treatment with Decalso, most of the activity passed into the filtrate. A mixture of the Decalso eluate and Decalso filtrate, however, gave enhanced growth equal to that given by the original chloroform extract. When Examen was treated with Decalso, most of the activity was present in the filtrate, though the eluate was more potent than

70% ethanol-insoluble precipitate (≡50 kg. liver) dissolved in water (6 l.), adjusted to pH 3 and filtered through charcoal (40 g.)

↓

Charcoal washed with 50% ethanol (2 × 400 ml.) and eluted by stirring with N-$NH_4OH$ in 50% ethanol (2 × 1 l.) at 60–70°

↓

Eluate evaporated to dryness, residue dissolved in water (400 ml.), and solution adjusted to pH 3

↓

Precipitate discarded and solution filtered through charcoal (5 g.)

↓

Charcoal washed with 50% ethanol (2 × 200 ml.) and eluted by stirring with N-$NH_4OH$ in 50% ethanol (2 × 500 ml.) at 60–70°

↓

Eluate evaporated to dryness and residue dissolved in water (500 ml.) = *Solution A*

↓

Solution A (250 ml.) treated with 5% $AgNO_3$ solution (40 ml.) and neutralized to pH 7 with NaOH

↓

Precipitate washed with water and decomposed with N-HCl (20 ml.)

↓

Solution adjusted to pH 3 and filtered through charcoal (4 g.)

↓

Charcoal eluted with N-$NH_4OH$ in 50% ethanol (2 × 200 ml.)

↓

Eluate evaporated to dryness and residue dissolved in 200 ml. of water

↓

Solution (150 ml.) adjusted to pH 4·5 and filtered through Decalso (100 g.; washed with 1% acetic acid); Decalso washed with hot water

| | |
|---|---|
| Decalso eluted with hot 10% NaCl solution (100 ml.) | Filtrate and washings (150 ml.) = *Solution B* |
| ↓ | |
| Eluate extracted with phenol, extract diluted with 5 vol. of ether and extracted with water = *Solution C* (50 ml.) | |

Fig. 1. Fractionation of the 70% ethanol-insoluble precipitate from Plexan.

Charcoal-Decalso eluate (*Solution C* (Fig. 1); 30 ml.)

| adjusted to pH 3 and extracted with chloroform (3 × 50 ml.) | | adjusted to pH 11 and extracted with chloroform (3 × 50 ml.) | |
|---|---|---|---|
| chloroform extract washed with water and extracted with 5% $NaHCO_3$ solution (3 × 20 ml.) | aqueous solution = *Solution E* | chloroform extract washed with water and extracted with N-HCl (3 × 20 ml.) | aqueous solution = *Solution G* |
| ↓ | | ↓ | |
| aqueous extract adjusted to pH 3 and extracted with phenol. Extract diluted with ether and extracted with water = *Solution D* (30 ml.) | | aqueous extract adjusted to pH 3 and extracted with phenol. Extract diluted with ether and extracted with water = *Solution F* (30 ml.) | |

Fig. 2. Fractionation by chloroform extraction of the charcoal-Decalso eluate from the 'Plexan' precipitate.

Table 1. *Relative potencies of fractions obtained from* 70% *(v/v) ethanol-insoluble precipitate from Plexan in stimulating the growth of* L. helveticus

| | Volume (ml.) of fraction added to 10 ml. of medium | | | | |
|---|---|---|---|---|---|
| | 0·0 | 0·1 | 0·5 | 1·0 | 2·0 |
| | Volume (ml.) of 0·1 N-NaOH required to neutralize the acid formed in 10 ml. medium | | | | |
| Solution A, Charcoal eluate | 0·3 | 8·0 | — | — | — |
| „ B, Decalso filtrate | 0·3 | 0·5 | 1·3 | — | — |
| „ C, Decalso eluate | 0·3 | 3·1 | 6·3 | — | — |
| „ D, Chloroform extract (pH 3) | 1·0 | 4·7 | 9·8 | 9·8 | 9·8 |
| „ E, Chloroform insoluble (pH 3) | 0·7 | 0·8 | 1·4 | 2·6 | 4·4 |
| „ F, Chloroform extract (pH 11) | 1·0 | — | 8·7 | 9·5 | 9·7 |
| „ G, Chloroform insoluble (pH 11) | 0·7 | 0·7 | 1·5 | 2·4 | 6·8 |

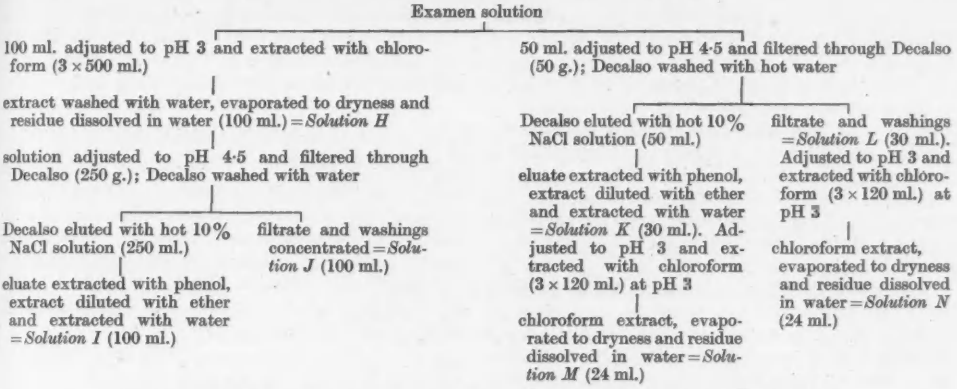
Examen solution
100 ml. adjusted to pH 3 and extracted with chloroform (3 × 500 ml.)
extract washed with water, evaporated to dryness and residue dissolved in water (100 ml.) = Solution H
solution adjusted to pH 4·5 and filtered through Decalso (250 g.); Decalso washed with water
Decalso eluted with hot 10% NaCl solution (250 ml.)
eluate extracted with phenol, extract diluted with ether and extracted with water = Solution I (100 ml.)
filtrate and washings concentrated = Solution J (100 ml.)
50 ml. adjusted to pH 4·5 and filtered through Decalso (50 g.); Decalso washed with hot water
Decalso eluted with hot 10% NaCl solution (50 ml.)
eluate extracted with phenol, extract diluted with ether and extracted with water = Solution K (30 ml.). Adjusted to pH 3 and extracted with chloroform (3 × 120 ml.) at pH 3
chloroform extract, evaporated to dryness and residue dissolved in water = Solution M (24 ml.)
filtrate and washings = Solution L (30 ml.). Adjusted to pH 3 and extracted with chloroform (3 × 120 ml.) at pH 3
chloroform extract, evaporated to dryness and residue dissolved in water = Solution N (24 ml.)

Fig. 3. Fractionation of Examen by adsorption on Decalso and extraction with chloroform.

Table 2. *Relative potencies of fractions obtained from Examen in stimulating growth of* L. helveticus *(measured by acid-production)*

| | Volume (ml.) of fraction added to 10 ml. of medium | | | |
|---|---|---|---|---|
| | 0·0 | 0·5 | 1·0 | 2·0 |
| | Volume (ml.) of 0·1 N-NaOH required to neutralize the acid formed in 10 ml. medium | | | |
| Solution H, Chloroform extract | 0·8 | 6·3 | 7·3 | 9·7 |
| „ I, Decalso eluate of chloroform extract | 0·1 | 0·7 | 1·0 | 1·4 |
| „ J, Decalso filtrate of chloroform extract | 0·1 | 2·6 | 3·9 | 6·1 |
| Solutions I + J | 0·1 | 5·7 | 6·8 | 9·9 |
| Solution K, Decalso eluate of Examen | 0·8 | 3·0 | 3·1 | 4·3 |
| „ L, Decalso filtrate of Examen | 0·8 | 6·3 | 8·5 | 9·9 |
| „ M, Chloroform extract of Decalso eluate | 0·8 | 3·0 | 3·1 | 4·2 |
| „ N, Chloroform extract of Decalso filtrate | 3·8 | 6·2 | 7·4 | 8·9 |

the eluate from the chloroform extract. The filtrate and eluate were then extracted with chloroform, and in both instances the extracts were active. These results appear to establish the presence of two different chloroform-soluble factors, one termed factor HL3 being present in the Decalso eluate, and the other, factor HL4, in the Decalso filtrate. They obviously differ from factor HL1 which, though soluble in chloroform and adsorbable on Decalso, is a more potent growth stimulant, and from factor HL2, which is chloroform-insoluble. It is possible, however, that factor HL1 is a mixture of factors HL3 and HL4.

*Action of reagents on the various growth factors*

*Acetylation.* The chloroform extract from Examen and the Decalso eluate and filtrate therefrom (solutions H, I and J) were acetylated, by adding to 15 ml. of each of the solutions, 2 ml. of 2N-NaOH and 0·2 ml. of acetic anhydride, repeating the addition after an interval of 2–3 min. The mixture was then left at room temperature for 15 min., neutralized and concentrated *in vacuo* to 15 ml. The activities of the solutions on *L. helveticus* and *S. lactis* R were unaffected by this treatment. The Decalso eluate and filtrate from Examen itself (solutions K and L) were also acetylated; again the activity was unaffected.

*Benzoylation.* Samples of solutions H, I and J (30 ml.) were shaken with benzoyl chloride (100 mg.) and 4N-NaOH (1·0 ml.) and left at room temperature for 1 hr. The mixture was acidified, extracted with ether to remove benzoic acid, and adjusted to pH 6. No loss of activity occurred. It is therefore concluded that factors HL3 and HL4 are not inactivated by benzoylation.

*Nitrous acid.* Samples (30 ml.) of solutions H, I and J were treated with $NaNO_2$ (100 mg.) and 2N-HCl (1·0 ml.); after standing at room temperature for 2 hr. each solution was treated with sulphamic acid (150 mg.) to destroy excess nitrous acid. After 10 min., the solutions were adjusted to pH 6. The activity on *L. helveticus* of the Decalso filtrate

from the chloroform extract (solution J) containing factor HL4 was markedly decreased, though not its activity for *S. lactis* R; however, its effect in presence of factor HL3 decreased for either organism.

*Effect of heat, acid and alkali.* The stability of factors HL3 and HL4 was tested under the following conditions: (*a*) refluxing solutions I and J for 3 hr.; (*b*) boiling for 3 hr. with 2N-NaOH; (*c*) boiling for 3 hr. with 2N-HCl. Neither of the factors was appreciably affected, whereas, according to Mitchell *et al.* (1941), folic acid is unstable under these conditions.

The effect of a mixture of factors HL3 and HL4 on *L. helveticus* was also tested on a medium in which the casein hydrolysate was replaced by a mixture of 16 amino-acids. Considerably less growth was obtained with this medium than with the original medium, suggesting that some additional factor may have been present in the casein hydrolysate (Table 3).

Table 3. *Effect of factors* HL3+HL4 *from Examen on the growth of* L. helveticus, *using medium containing casein hydrolysate or* 16 *amino-acids*

| | Volume (ml.) of solution added to 10 ml. of medium | | | | | |
|---|---|---|---|---|---|---|
| | 0·0 | 0·1 | 0·5 | 1·0 | 2·0 | 4·0 |
| | Volume (ml.) of 0·1N-NaOH required to neutralize acid produced | | | | | |
| Casein hydrolysate medium | 0·2 | 2·7 | 4·3 | 6·4 | 8·2 | — |
| Amino-acid medium | 0·2 | — | 0·9 | 1·3 | 3·0 | 5·5 |

An aqueous solution of the chloroform-soluble factors did not exhibit any characteristic absorption bands, but an aqueous solution of the chloroform-insoluble fraction from Examen gave an absorption spectrum similar to that obtained by Mitchell (1944) for folic acid, with a maximum at 267mμ and a minimum at 249mμ. These were displaced at pH 11 to 282 and 271mμ respectively.

## DISCUSSION

Assuming that factor HL2 is folic acid, the evidence now presented suggests the existence of two chloroform-soluble factors, each of which can replace folic acid as an essential growth factor for *L. helveticus* and *S. lactis*. The two factors can be separated by treatment with Decalso. The factor not adsorbed on Decalso (HL4) appeared to be more unstable to $HNO_2$ than the eluate factor (HL3).

## SUMMARY

1. Attempts have been made to prepare a concentrate of folic acid from by-products obtained in the manufacture of a commercial liver extract. In addition to a fraction that could not be extracted from aqueous solution by chloroform, other fractions soluble in chloroform and capable of stimulating the growth of *Lactobacillus helveticus* and *Streptococcus lactis* were obtained.

2. These fractions are believed to contain three or possibly four different growth factors. The chloroform-insoluble factor, designated factor HL2, is probably identical with folic acid. Factors HL1 and HL3 are soluble in chloroform and adsorbed on Decalso, but whereas HL1 is a potent growth-stimulant, HL3 is only slightly active. Factor HL4 is also soluble in chloroform and is a potent growth-stimulant, but it is not adsorbed on Decalso. Factor HL1 may be a mixture of factors HL3 and HL4. Factors HL3 and HL4 exert a synergistic action on one another.

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# Anaemia of Nutritional Origin in the Rat

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(*Received* 4 *May* 1945)

Many workers during the past ten years have reported the development in laboratory animals of anaemia resulting from deficiency in the diet of one or more components of the vitamin B complex. Rhoads & Miller (1933) reported an anaemia, macrocytic in type, in a small proportion of dogs maintained on a modified Goldberger diet, and suffering from recurrent attacks of black tongue. Cures were obtained following oral administration of meat, liver, or yeast. Wills & Stewart (1935) observed a macrocytic anaemia in monkeys fed on a diet low in its content of protein and the B vitamins. The anaemia responded to treatment with autolyzed yeast or a yeast filtrate fraction. Birch, Chick & Martin (1937) found that pigs fed on a modified Goldberger diet developed severe enteritis and a progressive anaemia. In later work Chick, Macrae, Martin & Martin (1938 *a*, *b*) found that the pellagra-like condition was rapidly cured by daily injections of nicotinic acid, though they did not state whether the blood picture responded to this treatment. More recently, Handler & Featherstone (1943) have reported that dogs developed a macrocytic anaemia with hyperplastic marrow about 60 days after remission of symptoms of black tongue following saline therapy. Following administration of nicotinic acid, these workers observed a progressive recovery from the anaemia, even though the food intake was restricted during the therapeutic period to a level similar to that of the period which preceded it. Apart from deficiency of nicotinic acid, many workers have provided evidence for the appearance of anaemias of different types and resulting from absence of other B vitamins in the diet. Chick *et al.* (1938*c*) clearly differentiated the effects of deficiency in the pig of yeast eluate and yeast filtrate fractions. On a synthetic ration supplemented by aneurin, riboflavin, nicotinic acid, and yeast filtrate fraction, their animals developed epileptiform convulsions and a microcytic anaemia. When the eluate fraction replaced the yeast filtrate in the diet, the clinical picture was of a flaccid hind limb paralysis together with a moderate normocytic anaemia. Wintrobe, Samter & Lisco (1939), Wintrobe, Miller, Follis, Stein, Mushatt & Humphreys (1942) and Wintrobe, Follis, Miller, Stein, Alcayaga, Humphreys, Suksta & Cartwright (1943) extended these observations on the pig. Using a synthetic ration in which brewer's yeast was subsequently replaced by supplements of aneurin, riboflavin, choline, nicotinic acid, and in some cases pantothenic acid, they reported the development of ataxia, sensory neurone degeneration, epileptiform fits and a microcytic anaemia. The last was characterized by polychromasia, hyperplasia of bone marrow, haemosiderosis in liver, spleen and marrow, and rise of serum iron level. Administration of pyridoxin was followed by reticulocytosis and blood regeneration. The existence of a microcytic hypochromic anaemia resulting from pyridoxin deficiency and cured by its administration has been similarly reported for the dog (Fouts, Helmer, Lepkovsky & Jukes, 1938; Fouts, Helmer & Lepkovsky, 1939, 1940; McKibbin, Madden, Black & Elvehjem, 1939–40; McKibbin, Scharfer, Frost & Elvehjem, 1942; Borson & Mettier, 1940). In the pyridoxin-deficient rat, Fouts & Lepkovsky (1942) observed a fall in haemoglobin less profound than in the dog. There have been suggestions that additional dietary factors may influence normal erythropoiesis. György, Goldblatt, Miller & Fulton (1937) claimed that a condition of panmyelophthisis with aplastic macrocytic anaemia developed in the rat after symptoms of acrodynia had been relieved by pyridoxin. György (1938) at first believed that inclusion of nicotinic acid in the diet prevented the onset of panmyelophthisis, but later (György & Eckhardt, 1940) this claim was withdrawn and was not confirmed by Chick, Macrae & Worden (1940). Borson & Mettier (1940) noted that after amelioration of microcytic anaemia in the dog with pyridoxin, there remained a residual mild normochromic anaemia which responded to large doses of an unknown factor present in a liver filtrate fraction.

A chick anti-anaemia factor, vitamin $B_c$, has been reported by Hogan & Parrott (1940), which has been identified with folic acid (O'Dell & Hogan, 1943). Campbell, Brown & Emmett (1944) confirmed this claim, and observed that presence of this factor in the diet gave protection, not only against fall of red cells, but also of thrombocytes and leucocytes. In the rat leucopenia and granulocytopenia induced by feeding sulphaguanidine and other sulphonamides are prevented by folic acid

concentrates (Nielsen & Elvehjem, 1942; Axelrod, Gross, Bosse & Swingle, 1943), and by crystalline folic acid (Daft & Sebrell, 1943). Langston, Darby, Shukers & Day (1940) have reported a nutritional anaemia and agranulocytosis which developed in rhesus monkeys fed on a modified Goldberger diet, and which failed to respond to nicotinic acid. The factor responsible for protection against this cytopenia in the monkey has been designated by these workers vitamin M. Wilson, Doan, Saslaw & Schwab (1942) found that intramuscular injections of a folic acid concentrate effected re-establishment of normal white cell equilibrium in the monkey. On the other hand, Totter, Shukers, Kolson, Mims & Day (1944) have failed to find any correlation between the folic acid content of the diet and its potency in protecting monkeys against nutritional cytopenia. They observed that xanthopterin, a fish anti-anaemic factor (Simmons & Norris, 1941), possessed vitamin M activity, although some of their animals appeared to show relapse during the course of treatment. The relationship between folic acid, xanthopterin and vitamin M remains uncertain. It is evident that many factors are involved in normal erythropoiesis.

The present work has for its object a further investigation of this problem in the rat. We have obtained evidence, which is presented in more detail below, that on diets which include an adequate intake of pyridoxin as well as of other factors needed by this species, a severe microcytic anaemia develops.

## METHODS

Sixty-six rats of the Wistar strain, weighing 40–50 g., were placed on the experimental diet shortly after weaning. Two simplified diets, which will be referred to as diet R and diet S, have been employed. Diet R had the following composition: rice starch 70%, caseinogen 20%, salt mixture 5%,* cod-liver oil 4%, agar 1%. Diet S was similar in composition to diet R, except that rice starch was replaced by sucrose. In control diets T and U, 15% dried brewer's yeast (Distillers Co. Ltd.) replaced equivalent amounts of starch or sucrose respectively. The starch and casein used in these experiments were twice extracted with 50% ethanol and twice with 96% ethanol over a period of a week, with frequent stirring. These diets were fed *ad libitum*, and each rat received daily supplements† of aneurin 20 $\mu$g., riboflavin 40 $\mu$g., pyridoxin 40 $\mu$g., choline chloride 500 $\mu$g., and in certain cases calcium-*d*-pantothenate 240 $\mu$g. Blood samples were obtained by tail bleeding, and haemoglobin estimations were performed by the Haldane method. Red and white cell counts were made at intervals during the course of the experiment.

* The salt mixture had the following composition: sodium chloride 4·69%, magnesium sulphate 7·22%, disodium hydrogen phosphate 9·21%, potassium dihydrogen phosphate 25·82%, calcium phosphate 14·62%, calcium lactate 35·22%, ferric ammonium citrate 3·15%, cupric sulphate 0·05%, potassium iodide 0·01%.

† Pyridoxin and riboflavin from Glaxo Laboratories Ltd., choline chloride from British Drug Houses Ltd., calcium-*d*-pantothenate from Roche Products Ltd.

## RESULTS

### *Signs of nutritional deficiency*

Figs. 1–4 illustrate the growth curves in a typical experiment, in which 15 rats, shortly after weaning, were distributed as follows: Group M 1 (5 rats, nos. 71, 72, 78, 79, 85) received diet S, together with daily supplements of aneurin 20 $\mu$g., riboflavin 40 $\mu$g., pyridoxin 40 $\mu$g., choline chloride 500 $\mu$g., and calcium pantothenate 240 $\mu$g. Group M 2 (5 rats, nos. 73, 74, 80, 81, 86) received diet S and similar supplements, except for the omission of pantothenate. Group M 3 (5 rats, nos. 75, 76, 82, 83, 87) received diet U containing 15% yeast, together with the supplements as for group M 1. There seems to be no marked difference in the growth response of groups M 1 and M 3, though the maximum plateau was in each case lower than that reached by similar rats (group M 4, nos. 77, 84, 88) on a stock laboratory diet of commercial rat cake and milk. The growth response of the animals in group M 2, deficient in pantothenic acid, was much inferior, and weight became stabilized at about 100 g. In other experiments a terminal fall of weight followed by death was frequently observed unless early treatment with pantothenic acid was begun. The most conspicuous external evidences of deficiency were those which have been attributed to lack of pantothenic acid. These features, which have also been noted by other workers (Unna & Sampson, 1940; Daft, Sebrell, Babcock & Jukes, 1940; Figge & Salomon, 1941–2; Chick *et al.* 1940) and which usually appeared in our rats within 6–8 weeks, included an incrustation of the whiskers and hair of the eyes, nose, and paws with a porphyrin which fluoresced a vivid red in ultra-violet light. The abdominal hair was matted and stained brown, possibly owing to an inability of the animals to clean themselves effectively. In some cases alopecia was a prominent feature. This may be related to absence of inositol in the diet (Woolley, 1941). The only source of fatty acids in the diet was cod-liver oil. Other fat was deliberately excluded in order to avoid possible introduction of traces of B vitamins, and this may have been responsible for the scaliness and patchy dermatitis of the tail which frequently occurred. No symptoms of motor ataxia were observed.

### *Anaemia*

In the assessment of the incidence of anaemia in the rat, it is important to determine the changes in the blood picture which occur during the early period of growth of the animal. The normal blood picture has been established in a series of 12 male

Table 1. *Development of anaemia in rats on various diets*

(Data are given for one rat from each group.)

| Group | No. rats in group | Rat-identifying numbers | Diet | Days on diet | Red blood cells ($10^6$/cu.mm.) | Haemoglobin index | Colour index | Reticulocytes (no./100 red blood cells) |
|---|---|---|---|---|---|---|---|---|
| M1 | 5 | 85 | Diet S + all vitamin supplements* | 0 | 5·05 | 80 | 0·78 | 19·2 |
| | | | | 52 | 8·49 | 108 | 0·64 | 2·4 |
| | | | | 101 | 7·98 | 98 | 0·61 | 1·4 |
| | | | | 112 | 6·44 | 84 | 0·65 | 1·4 |
| | | | | 167 | 4·21 | 60 | 0·62 | 6·8 |
| | | | | 188 | 4·98 | 64 | 0·64 | 1·2 |
| M2 | 5 | 74 | Diet S + vitamin supplements except pantothenate* | 0 | 5·56 | 84 | 0·75 | 11·2 |
| | | | | 52 | 8·04 | 100 | 0·62 | 4·2 |
| | | | | 101 | 6·60 | 78 | 0·60 | 1·6 |
| | | | | 112 | 6·40 | 74 | 0·60 | 4·3 |
| | | | | 146 | 2·51 | 15 | 0·30 | 64·0 |
| M3 | 5 | 83 | Diet U + all vitamin supplements* | 0 | 5·96 | 82 | 0·68 | 9·4 |
| | | | | 54 | 9·05 | 112 | 0·61 | 3·4 |
| | | | | 102 | 8·54 | 104 | 0·61 | 2·2 |
| | | | | 139 | 8·10 | 100 | 0·59 | 0 |
| M4 | 3 | 77 | Rat cake + milk | 0 | 6·70 | 90 | 0·67 | 6·8 |
| | | | | 54 | 8·51 | 108 | 0·63 | 1·8 |
| | | | | 102 | 8·08 | 98 | 0·58 | 3·2 |
| | | | | 139 | 8·10 | 96 | 0·60 | 0·8 |

* Vitamin supplements: aneurin 20 $\mu$g., riboflavin 40 $\mu$g., pyridoxin 40 $\mu$g., choline chloride 0·5 mg., calcium pantothenate 240 $\mu$g.

and 7 female animals placed after weaning on the control diets T and U, or the stock laboratory diet. Blood counts and haemoglobin estimations were performed at intervals over a period of 6–8 months. At 40–50 g. weight the red cell count and haemoglobin index average 5·5 million/cu.mm. and 80% respectively, and thereafter rise linearly to reach an adult mean of $8·60 \pm 1·90$ million/cu.mm. and $107 \pm 17·5$. This mean is the same for males and females. It is reached at a weight range of 140–150 g. for males and 110–120 g. for females, at about 3 months of age. In the case of animals on a deficient diet the onset of anaemia is a late feature, and in all cases in which it was observed there was an initial rise both of red cell count and of haemoglobin index from the level characteristic of the young animal to the mean seen in the normal at 110–150 g. For our present purpose anaemia is defined as a fall in the erythrocyte count and haemoglobin index below the level of the mean minus $3\sigma$ observed in normal animals of the same age group.

Table 1 and Figs. 1–4 record the blood findings for each group in the typical experiment referred to above. None of the control animals in group M3 on a diet containing 15% yeast, together with all vitamin supplements, nor those in group M4 on a stock laboratory diet, developed anaemia during the period in which they were under observation.

Three animals in group M1 on diet S, together with vitamin supplements including pantothenic acid, developed a severe anaemia after 188 days. The remaining two animals of this group showed a slight fall of haemoglobin index at the 146th and 181st day respectively, when they were sacrificed. The changes in the morphology of the blood cells were much less marked than those associated with pantothenic acid deficiency described below. In severe cases there was polychromasia, but little anisocytosis, and no evidence of granulopenia. In less severe cases the morphology seemed normal. The bone marrow was hyperplastic but orthoplastic, and showed no evidence of failure of maturation. The significance of this anaemia is discussed later.

Table 2. *Incidence of anaemia in rats receiving, and deficient in, pantothenate*

| | No. of rats |
|---|---|
| Animals deficient in pantothenic acid: | |
| (*a*) Surviving less than 7 weeks | |
| Anaemic | 0 |
| Not anaemic | 7 |
| (*b*) Surviving longer than 7 weeks | |
| Anaemic | 24 |
| Not anaemic | 11 |
| Animals receiving pantothenic acid: | |
| (*a*) Surviving less than 7 weeks | 0 |
| (*b*) Surviving longer than 7 weeks | |
| Anaemic | 5 |
| Not anaemic | 0 |
| Controls on normal diets: | |
| Anaemic | 0 |
| Not anaemic | 19 |

Three rats in group M2, deficient in pantothenic acid, developed a severe anaemia in 58, 101 and 112 days respectively. The remaining two animals

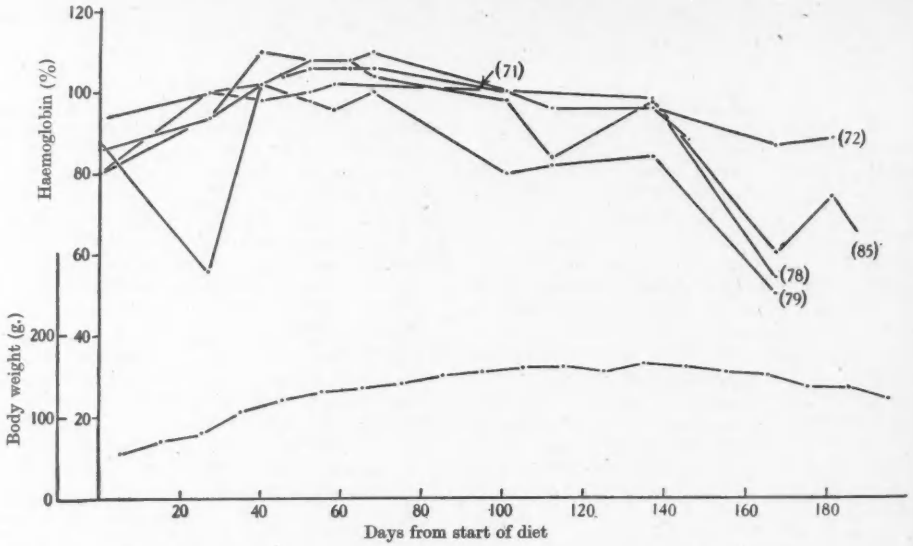



Fig. 1. Changes in body weight and haemoglobin index on diet S together with all vitamin supplements. The lower curve gives the average body weight of the group. The number identifying each rat is given in brackets.

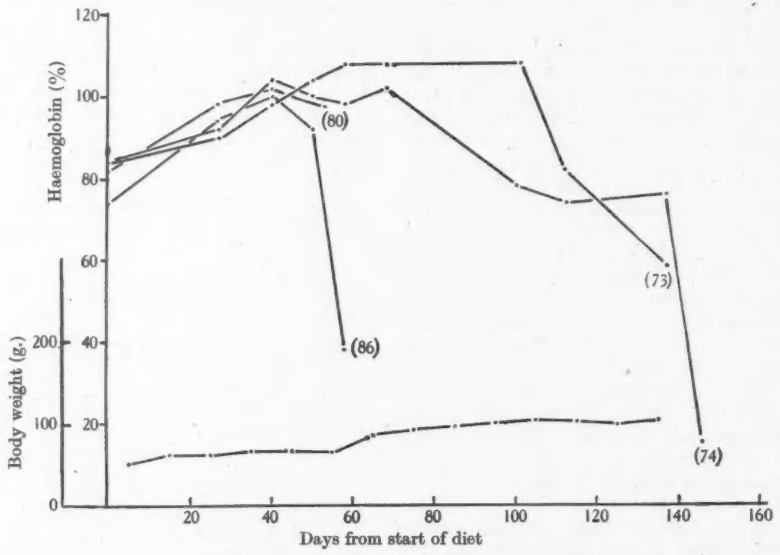



Fig. 2. Changes in body weight and haemoglobin index on diet S together with all vitamin supplements except pantothenic acid. The lower curve gives the average body weight of the group. The number identifying each rat is given in brackets. One rat of this group, no. 81, whose haemoglobin curve is not given, died and had a haemoglobin index of 82%.

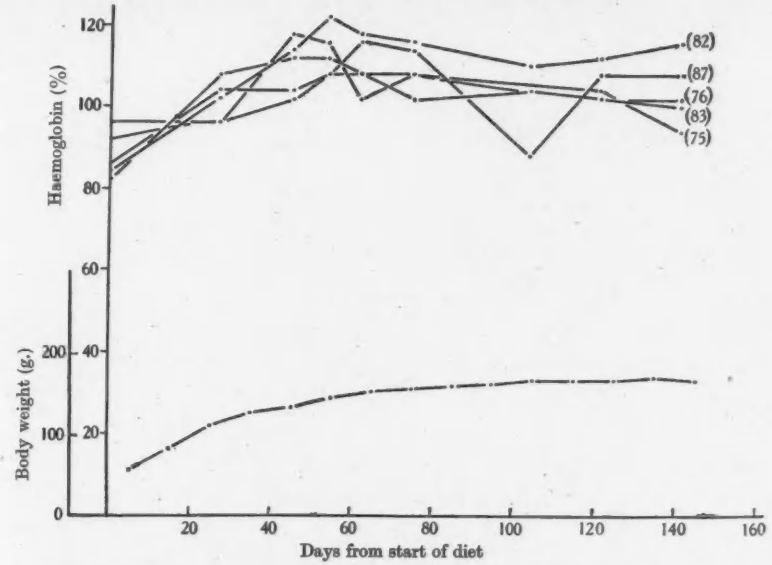



Fig. 3. Changes in body weight and haemoglobin index on diet U together with all vitamin supplements. The lower curve gives the average body weight of the group. The number identifying each rat is given in brackets.

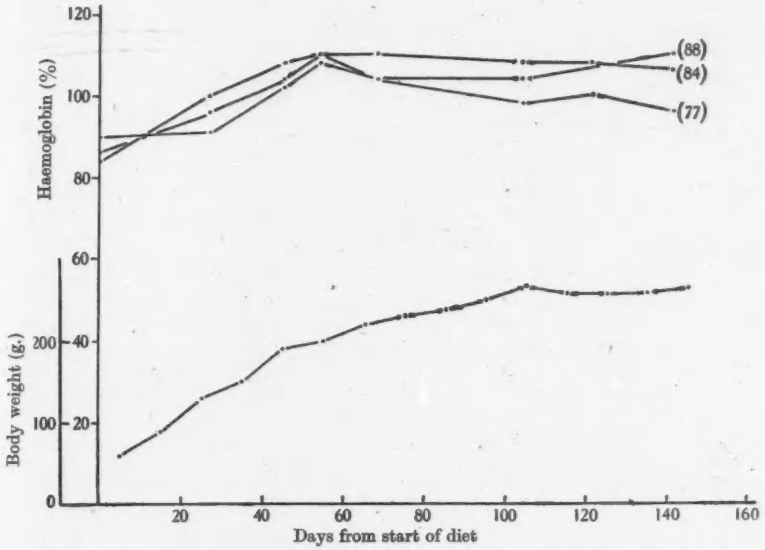



Fig. 4. Changes in body weight and haemoglobin index on diet of rat cake and milk. The lower curve gives the average body weight of the group. The number identifying each rat is given in brackets.

of this group died after 25 and 57 days respectively, without anaemia. In all we have had 42 rats on pantothenic acid-deficient diets R and S (Table 2). Of these animals, seven survived for less than 7 weeks, and none became anaemic. Thirty-five rats survived for a period longer than 7 weeks; of these 66% developed anaemia. The first change in the blood morphology associated with deficiency of pantothenic acid appears to be an increase of polychromasia as compared with control animals of the same age. This was followed by anisocytosis as the anaemia developed. The final stage was characterized by extreme anisocytosis, polychromasia and poikilocytosis, together with the presence of target cells, polychromatic or even basophilic normoblasts, and by polymorphonuclear leucopenia verging on agranulocytosis. The bone marrow, details of which are described in detail below, showed extreme hyperplasia and evidence of arrested maturation of both erythropoietic and leucopoietic cells (Pl. 4).

*Curative tests with pantothenic acid.* In the prophylactic experiment described above, the onset of anaemia appears to be delayed in those animals receiving pantothenic acid as compared with animals deficient in this factor. Thus the average time of onset of anaemia in animals of group M 1 receiving daily supplements of pantothenic acid was 172 days, whereas the average time for pantothenic acid-deficient animals was 74 days. Too much emphasis must not be placed on this difference at present in view of the small number of animals investigated. Nevertheless it seemed of interest to study the curative effect of pantothenic acid in anaemic animals deficient in this factor. Twelve rats, which had developed anaemia of varying severity under conditions previously described, were given daily supplements of 240 $\mu$g. calcium pantothenate for periods up to 80 days (Table 3). Certain other animals which had become severely anaemic and which had died within 3 days of the commencement of treatment are not included in this series. Three rats, nos. 6, 55 and 65, survived, and a normal blood picture was restored in two cases within 3 weeks, but in no. 6 only after some 11 weeks. No. 50 showed gradual but complete blood regeneration after 10 weeks but subsequently died, while no. 48 showed partial recovery but died before this was complete. Seven rats died without any evidence of recovery from anaemia, but in four of these death occurred within 8 days from the commencement of pantothenate administration. Our present experience is that it is necessary to begin treatment at an earlier stage in the development of anaemia if it is to be successful.

### *Morbid anatomy and histology*

The morphology of the organs was studied in eighteen animals of the present series. Of this group ten died or were killed at the end of the experiment as a consequence of an outbreak of salmonella infection in another group of rats which spread to our animals. No conclusion as to the significance of the morphological changes seen in the infected animals can be drawn, and they will not be considered further. The remaining eight rats included three animals (nos. 16, 36 and 45) which were normal controls, and one anaemic animal, no. 71, on the synthetic diet with pantothenic acid. Nos. 58, 73, 74 and 86 were on a pantothenic acid-deficient diet and were all anaemic.

#### A. *Pantothenic acid-deficient animals*

All were subnormal in weight and showed reddish brown staining of the hair of the abdominal wall and nares with scaliness of legs and tail. There was marked pediculosis. Marked absence of omental and depot fat.

*Skin.* Marked hyperkeratosis and moderate parakeratosis. No acanthosis, but proliferation of basal cells and irregularity of rete pegs. There were collections of neutrophils in the hyperkeratotic plaques, and areas of ulceration and small subepithelial abscesses.

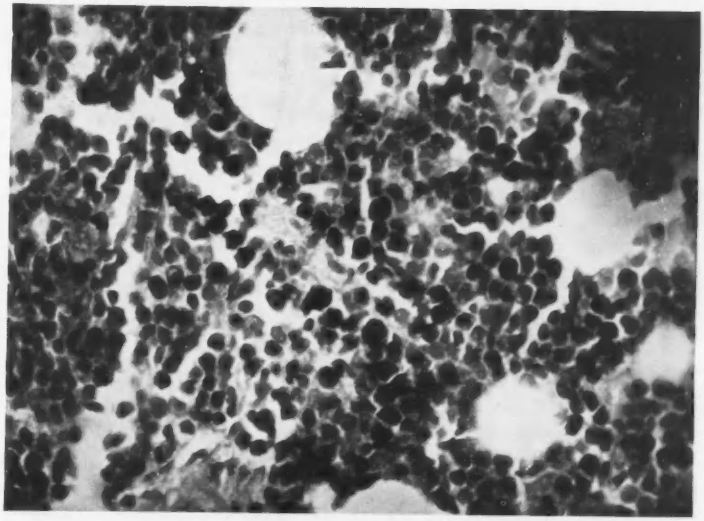
*Skeletal muscle.* Degenerative changes with focal loss of striation and proliferation of sarcolemmal nuclei. These features were also seen in animals receiving pantothenic acid, and may be attributed to general undernutrition rather than to any specific deficiency.

*Liver.* No abnormality.

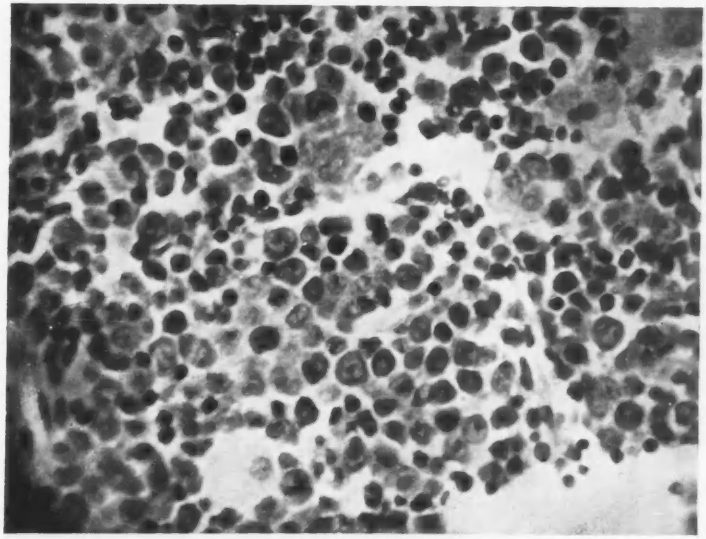
*Spleen.* Marked myeloid transformation, both leucopoietic and erythropoietic cells being present. Many of the myeloid and erythroid cells showed the failure of nuclear maturation seen in the marrow. Moderate siderosis. No fibrosis. The myeloid transformation was not observed in the control animals.

*Bone marrow.* In three animals (nos. 73, 74 and 86) there was extreme hyperplasia in the marrow of femur and ribs, with almost complete absence of fat spaces, and failure of maturation in both leuco- and erythropoietic cells. Polychromatic and orthochromatic erythroblasts with primitive leptochromatic nucleus, and many cells of the pro-erythroblast series were present. In rat 58, which was severely anaemic, there was marked hypoplasia of marrow, with fatty replacement. There were a few islands of basophil erythroblasts, isolated primitive mesenchymal cells, and very few granular cells, though mast cells were prominent. The vertebral marrow of the tail showed no hyperplasia in any of these animals (Pl. 4).

*Gonads.* Spermatogenesis absent in contrast to the controls and to those pantothenic acid-deficient animals which survived less than 7 weeks. The ovaries were hypoplastic, but there appeared to be normal ripening of the follicles.



(*a*)



(*b*)

(*a*) Bone marrow of normal rat on diet of rat cake and milk. Section shows normal degree of cellularity in which fat spaces are present. A high proportion of the cells are mature in type—myelocytes, granulocytes, and orthochromatic normoblasts. Magnification, ×520. Barrett's stain. (*b*) Bone marrow of anaemic rat on pantothenic acid-deficient diet. Section shows considerable hyperplasia with absence of fat spaces, and the cells show failure of maturation both of the erythroid and myeloid series. The increase in cell size and immature type of nucleus can be clearly seen. Magnification, ×520. Barrett's stain.



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Table 3. *Effect of administration of pantothenic acid on anaemia*

| Rat no. | Days on pantothenate-deficient diet | Days on panto-thenate treatment | Red blood cells ($10^6$/cu.mm.) | Haemo-globin index | Colour index | Remarks |
|---|---|---|---|---|---|---|
| 6 | 195 | — | 7·41 | 100 | 0·67 | |
| | 223 | — | 7·48 | 86 | 0·57 | |
| | 278 | — | 3·90 | 50 | 0·64 | |
| | — | 18 | 6·41 | 48 | 0·37 | |
| | — | 28 | 7·66 | 58 | 0·37 | |
| | — | 44 | 7·79 | 68 | 0·43 | |
| | — | 80 | 8·03 | 94 | 0·58 | Cured. Survived |
| 55 | 62 | — | 8·30 | 116 | 0·75 | |
| | 76 | — | 7·63 | 104 | 0·68 | |
| | 85 | — | 5·35 | 72 | 0·66 | |
| | — | 6 | 4·90 | 74 | 0·75 | |
| | — | 22 | 8·30 | 104 | 0·63 | Cured. Survived |
| 65 | 14 | — | 8·34 | 104 | 0·61 | |
| | 57 | — | 6·28 | 78 | 0·61 | |
| | — | 22 | 8·15 | 100 | 0·61 | Cured. Survived |
| 50 | 40 | — | 7·98 | 78 | 0·49 | |
| | — | 14 | 6·11 | 68 | 0·47 | |
| | — | 25 | 6·05 | 66 | 0·55 | |
| | — | 33 | 7·59 | 78 | 0·51 | |
| | — | 73 | 8·18 | 112 | 0·67 | |
| | — | 107 | — | — | — | Cured. Subsequently died |
| 48 | 57 | — | 8·23 | 102 | 0·65 | |
| | 68 | — | 7·14 | 86 | 0·60 | |
| | 76 | — | 6·07 | 69 | 0·57 | |
| | — | 38 | 7·38 | 72 | 0·49 | Improved. Subsequently died |
| | — | 48 | — | — | — | |
| 3 | 136 | — | 8·79 | 98 | 0·56 | |
| | 154 | — | 3·72 | 42 | — | |
| | — | 8 | — | — | — | No improvement. Died |
| 4 | 81 | — | 8·89 | 110 | 0·62 | |
| | 154 | — | 5·73 | 52 | 0·45 | |
| | — | 7 | — | — | — | No improvement. Died |
| 49 | 40 | — | 8·22 | 78 | 0·47 | |
| | — | 14 | 5·73 | 48 | 0·45 | |
| | — | 21 | — | — | — | No improvement. Died |
| 51 | 63 | — | 8·26 | 110 | 0·66 | |
| | 91 | — | 8·07 | 98 | 0·60 | |
| | — | 10 | 7·10 | 86 | 0·60 | |
| | — | 26 | 2·69 | 24 | 0·44 | |
| | — | 29 | — | — | — | No improvement. Died |
| 54 | 63 | — | 8·10 | 106 | 0·65 | |
| | 91 | — | 6·98 | 86 | 0·61 | |
| | — | 26 | 3·70 | 38 | 0·51 | |
| | — | 29 | — | — | — | No improvement. Died |
| 67 | 59 | — | 6·25 | 76 | 0·61 | |
| | — | 5 | — | — | — | No improvement. Died |
| 70 | 37 | — | 7·70 | 110 | 0·68 | |
| | 55 | — | 5·89 | 84 | 0·70 | |
| | — | 6 | — | — | — | No improvement. Died |

*Adrenals.* No evidence of necrosis or haemorrhage.

B. *Animals receiving pantothenic acid*

Well nourished, with ample depot fat. Coat in good condition. No splenomegaly, nor myeloid transformation. The marrow was hyperplastic and orthoplastic, but did not show the marked failure of maturation seen in the pantothenic acid-deficient animals.

## DISCUSSION

The evidence presented above indicates the development in the rat, under the conditions stated, of two types of nutritional anaemia differing in their blood morphology and in their biochemical origin. The first of these anaemias, about which we have at present most evidence, is associated with deficiency of pantothenic acid. It occurs, in conjunction with other manifestations of its deficiency, in about 60 %

of cases. It develops within 8–16 weeks, and in a certain proportion of cases is cured by administration of pantothenic acid. It is characterized by a rapid fall of haemoglobin and of erythrocytes and polymorphonuclear leucocytes. In the final stage there is extreme polychromasia, anisocytosis and poikilocytosis. Post-mortem the most striking changes are splenomegaly with myeloid transformation, and, in three of the four cases examined, hyperplasia in the marrow of ribs and femur with failure of maturation in both erythropoietic and leucopoietic cells. Administration of pantothenic acid resulted in restoration of a normal blood picture in only 25% of the animals treated, but our present experience suggests that failure to improve the condition was, in some cases, due to the fact that treatment was begun too late. The morphology of the red cells seen in our animals deficient in pantothenic acid appears to be very similar to that described by Wintrobe *et al.* (1943) in pyridoxin-deficient pigs. On the other hand, the polymorphonuclear leucopenia, verging on agranulocytosis in the final stage, is a feature reminiscent of folic acid deficiency seen in rats treated with sulphonamides. Our animals received no preparation of folic acid, and the question of its lack as a contributory factor in the development of this anaemia is therefore raised. On the other hand, the curative effect of pantothenic acid in certain cases is definite. The extreme degree of reticulocytosis would, in human subjects, be suggestive of a haemolytic origin. However, these features are also seen in pyridoxin deficiency in the pig, in which Wintrobe appears to have excluded haemolysis. It may be noted that we have observed no evidence of bilirubinaemia. The significance of reticulocytosis in the rat is not yet clear, but there seems no doubt that pantothenic acid deficiency leads to a progressive interference with erythropoiesis and leucopoiesis.

In contradistinction to the picture described above is that of rats which received pantothenic acid throughout the experimental period and which also became anaemic. Changes in the morphology of the blood cells were confined to polychromasia and there was little anisocytosis and no evidence of granulocytopenia. The marrow showed moderate hyperplasia, but no indication of maturation arrest. In view of the small number of animals receiving pantothenic acid throughout the experiment, and also of the fact that as the present work was nearing completion an outbreak of infection in a group of rats unconnected with our experiment spread to some of our animals and led to the temporary suspension of the investigation, it would be inexpedient to draw definite conclusions as to the significance of this anaemia. Further work which is confirmatory of these findings is still in progress.

## SUMMARY

1. Rats suffering from deficiency of pantothenic acid develop a severe hypochromic anaemia in about 60% of cases.

2. The anaemia is characterized by fall of haemoglobin, erythrocytes and polymorphonuclear leucocytes. The erythrocytes show marked polychromasia, anisocytosis and poikilocytosis.

3. Post-mortem there is splenomegaly with myeloid transformation. The bone marrow usually shows hyperplasia, and evidence of failure in maturation of both erythropoietic and leucopoietic cells. No pathological changes in the adrenal cortex were observed.

4. Administration of pantothenic acid results in a restoration of a normal blood picture in a proportion of cases, but it is important that treatment should begin at an early stage in the development of anaemia if it is to be successful.

5. An anaemia, differing in morphology from that described above, develops at a later stage in animals receiving pantothenic acid throughout the experimental period. The significance of this anaemia is at present uncertain.

We desire to thank Prof. R. A. Peters, F.R.S., for his interest in this work. Our thanks are also due to Miss Kempson for her care of the animals.

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# The Inhibition of Amine Oxidase by Amidines

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(*Received* 25 *May* 1945)

The enzymic oxidation of diamines such as putrescine, cadaverine or histamine by extracts from pig's kidney was found to be somewhat inhibited by guanidine (Blaschko, 1938, 1939), and Zeller (1938) added the observation that some guanidine derivatives, including synthalin (decamethylene diguanidine), acted as more powerful inhibitors. The trypanocidal action of synthalin was the starting-point of Yorke's (1940) work on diamidines. The action of amidine derivatives on the amine oxidases was therefore more fully studied.

The present paper gives our results with amine oxidase only; the observations on diamine oxidase will be reported separately.

## METHODS

Rabbit liver was used throughout. The tissue was ground with sand and its own weight of distilled water then added. After centrifuging, the supernatant fluid was dialyzed against distilled water overnight and one-tenth volume of 0·2 M-sodium phosphate buffer pH 7·4 was added. The substrate used was tyramine. The rate of oxidation was followed manometrically. Open manometers and conical flasks fitted with a side-bulb and a centre tube containing 0·3 ml. N-KOH were used: the gas phase was oxygen and the temperature 38°.

For each experiment four vessels (I–IV) were used, each containing 0·5 ml. extract + 1·1 ml. M/15 sodium phosphate pH 7·5, made up to a total of 2 ml. with water or addition. The additions were: nos. II and IV, 0·2 ml. 0·1 M-tyramine hydrochloride; nos. III and IV, 0·2 ml. inhibitor. The inhibitor concentration varied according to the activity of the compound. Final concentrations above $10^{-3}$ M were not used. The experiments were carried on for 30 or 45 min. and readings were taken every 15 min. For the calculation of the percentage inhibition the $O_2$ uptake during either the first 15 or 30 min. was taken, whichever was nearer to 100 $\mu$l. in vessel II. In the great majority of the experiments there was no significant difference in the percentage inhibition found at the end of the different periods.

The substances used in these experiments were kindly given to us by Dr A. J. Ewins, F.R.S., and Dr H. King, F.R.S., Boots Pure Drug Co. Ltd., provided those substances previously studied by Dr W. A. Broom (1936). The substances labelled V147, V187 and V335 and 'Marfanil' (p. 349) we obtained through the kindness of Dr J. Walker. Unless stated otherwise the substances were used as hydrochlorides (or dihydrochlorides). Tetramethylene diguanidine (arcaine) sulphate was a commercial sample from Roche Products Ltd.; for the synthalin B (dodecamethylene diguanidine dihydrochloride) we are indebted to Dr E. M. Lourie.

## EXPERIMENTS

### (1) *Monoamidines of the type* $CH_3.(CH_2)_n.C(:NH).NH_2$

Six representatives of this group were examined, four of which were studied by Broom (1936); in these $n$ was 3, 4, 8 and 10 respectively. In addition we had from Dr King two substances in which $n$ was 14 and 16. The results are shown in Table 1.

Compared with some of the amidines to be discussed later, the inhibitions observed were not very strong. The percentage inhibition rose with an increase of the length of the carbon chain up to the substance with 10 $CH_2$ groups, but fell off rapidly with the longer chains of 14 and 16.

Table 1. *Inhibition of amine oxidase by monoamidines of the type* $CH_3(CH_2)_nC(:NH)NH_2$

(Inhibitor concentration $10^{-3}$ M)

| $n$ | Inhibition (%) | $n$ | Inhibition (%) |
|---|---|---|---|
| 3 | 15 | 10 | 61 |
| 4 | 42 | 14 | 8 |
| 8 | 50·5 | 16 | 7·5 |

### (2) *Diamidines of the type* $NH_2(NH:)C(CH_2)_nC(:NH)NH_2$

In this series the number of compounds at our disposal was greater. The substance with 7 $CH_2$ groups, azelamidine, is one of Broom's substances and we obtained from Dr King the diamidines with 8, 9, 10, 11, 12, 13, 14, and 16 $CH_2$ groups. There were great differences in the inhibitory activity of the members of this series; two different diamidine concentrations had, therefore, to be employed; the short-chain members, which were relatively weak

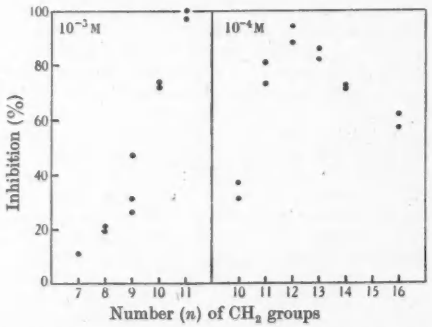
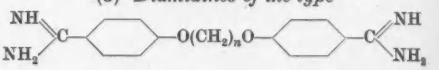



Fig. 1. Inhibition of amine oxidase by straight-chain diamidines $NH_2(NH:)C(CH_2)_nC(:NH)NH_2$. Inhibitor concentrations as shown. Each dot represents one experiment.

inhibitors, were used in a concentration of $10^{-3}$ M, those with higher molecular weights in $10^{-4}$ M. The results are shown in Fig. 1. It can be seen that the inhibitor activity rises steadily from $n=7$ to $n=12$ and with increasing chain length falls again.

### (3) *Diamidines of the type*



This group included the two trypanocidal drugs, propamidine ($n=3$) and pentamidine ($n=5$). We were given the substances with 1–6 $CH_2$ groups and the compound with 10 $CH_2$ groups by Dr Ewins; of these, the substances where $n$ was 3, 5, 6 and 10 were the di-isethionates (isethionic acid is 2-hydroxyethane-1-sulphonic acid).

Some of the substances in this group proved to be very powerful inhibitors of the enzyme; Fig. 2

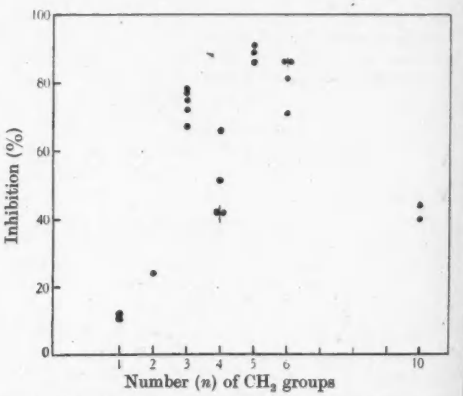
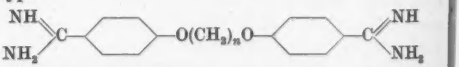



Fig. 2. Inhibition of amine oxidase by diamidines of the type



Inhibitor concentration $10^{-5}$ M. Each dot represents one experiment.

shows the effect of $10^{-5}$ M inhibitor. The strongest inhibitors were propamidine and pentamidine, the intermediary substance with 4 $CH_2$ groups was less active.

Three methods were employed to decide if the pentamidine inhibition was reversible:

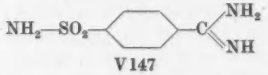
(*a*) When the extract together with pentamidine was dialyzed for 24 hr. against water the inhibitory effect of the pentamidine was not removed.

(*b*) Pentamidine gives a precipitate with salts of dibasic acids, e.g. sodium oxalate and sodium succinate; an experiment was therefore set up in which the inhibition by $10^{-5}$ M-pentamidine was examined in the presence and in the absence of $10^{-2}$ M-sodium oxalate. There was no difference in the percentage inhibition.

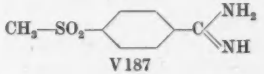
(*c*) By prolonged centrifugation and repeated washing (three times) with M/15 phosphate buffer a completely insoluble preparation of amine oxidase can be obtained from the rabbit's liver (Bernheim & Bernheim, 1938). Pentamidine in $10^{-5}$M concentration was added to such an enzyme preparation and the suspension was then repeatedly washed with phosphate buffer and centrifuged: after four washings there was no certain evidence that the pentamidine inhibition had been diminished in intensity, but after eight successive washings the inhibition had been noticeably reduced (from 94 to 66%). This showed that the inhibition was at least partly reversible, but that the diamidine was very firmly held by the enzyme.

(4) *Other amidines*

(*a*)

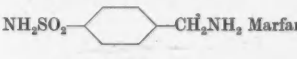


V147

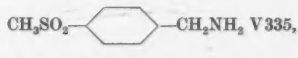


V187

The two substances V147 and V187 were tested in $10^{-3}$M concentration: the former caused about 57% inhibition, the latter none. It is interesting that these two amidines had little or no affinity for amine oxidase, although the corresponding amines (Marfanil and V335),



Marfanil,



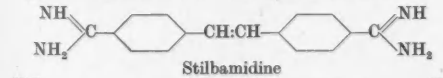
V335,

were readily oxidized as was shown by comparison with benzylamine (all substrates $10^{-2}$M; rabbit liver extract).

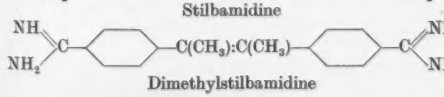
| Duration (min.) | $O_2$ uptakes in excess of blank (μl.) With benzylamine | With V335 | With Marfanil |
|---|---|---|---|
| 15 | 33 | 17 | 6 |
| 30 | 47 | 26 | 12 |
| 45 | 58 | 32 | 16 |

This experiment confirms a suggestion made by Evans, Fuller & Walker (1944) that the two amines might be substrates of amine oxidase. The oxidation of Marfanil by guinea-pig liver has recently been reported by Beyer & Govier (1945).

(*b*) *Stilbamidine and dimethylstilbamidine*



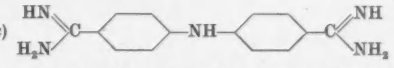
Stilbamidine



Dimethylstilbamidine

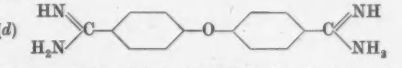
These were tested in $10^{-3}$M concentration. The inhibitions in one experiment were 44% for stilbamidine and 55% for dimethylstilbamidine. In two more experiments stilbamidine gave inhibitions of 24 and 34%. The stilbamidine was used as the diisethionate, whilst the dimethylstilbamidine was the dihydrochloride.

(*c*)



The dihydrochloride ($10^{-3}$M) gave 33% inhibition.

(*d*)



Phenamidine ($10^{-3}$M) inhibited only 15 and 25% respectively, in two experiments.

(5) *Monoguanidines of the type* $CH_3(CH_2)_nNHC(:NH)NH_2$

Only the compounds where $n = 8$ (the hydriodide) and $n = 13$ (the hydrochloride) were tested ($10^{-3}$M). Two additional substances with $n = 15$ and $n = 17$ were not sufficiently soluble. The inhibitions were 49% ($n = 8$) and 32% ($n = 13$).

(6) *Diguanidines of the type* $NH_2C(:NH)NH(CH_2)_nNHC(:NH)NH_2$

Fig. 3 gives the results for the straight chain diguanidines ($10^{-3}$M). There was a rise of inhibitor activity up to the substance with 14 $CH_2$ groups, whilst that with 18 $CH_2$ seemed a little less active (at a concentration of $10^{-4}$M the figures were 43% for the substance $n = 14$ and 39% for $n = 18$).

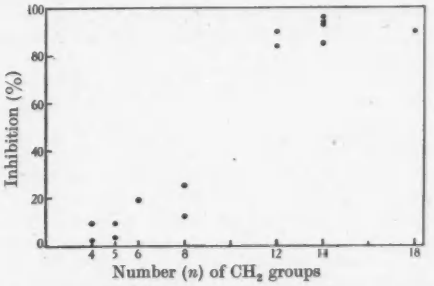



Fig. 3. Inhibition of amine oxidase by diguanidines of the type $NH_2C(:NH)NH(CH_2)_nNHC(:NH)NH_2$. Inhibitor concentration $10^{-3}$M. Each dot represents one experiment.

In addition a spermine derivative,

$NH_2C(:NH)NH(CH_2)_3NH(CH_2)_4$
$NH(CH_2)_3NHC(:NH)NH_2 . 4HCl$,

was examined. Its inhibitory activity was relatively low (17% in $10^{-3}$M concentration).

(7) *Diisothioureas of the type* $NH_2(NH:)CS(CH_2)_nSC(:NH)NH_2.HBr$

The substances, obtained from Dr King, were those with 2, 3, 6, 10 and 12 $CH_2$ groups. The results are shown in Table 2. Of the substances examined the compound with 10 $CH_2$ groups was most active.

Table 2. *Inhibition of amine oxidase by diisothioureas of the type* $NH_2(NH:)CS(CH_2)_nSC(:NH)NH_2$

| | Inhibition (%) | |
|---|---|---|
| n | $10^{-3}$M | $10^{-4}$M |
| 2 | 0 | — |
| 3 | 0 | — |
| 6 | 31 | — |
| 10 | 100 | 87 |
| 12 | 93 | 68 |

## DISCUSSION

The experiments described show that amidines have an affinity for amine oxidase, but their affinity, as measured by their inhibitory activity, depends considerably upon the general configuration of the substances examined. This is not surprising. It is known that not all amines react with amine oxidase; here too the molecular configuration determines affinity.

Inhibitors are usually more suitable for the study of the effects of molecular groupings on affinity than are substrates, as the rate of reaction is partly determined by other factors. The results obtained with the monoamidines and diamidines allow the following conclusions:

(1) In each series there is an increase in inhibitory activity with an increasing number of $CH_2$ groups until a certain chain length is reached; beyond this point a further increase in chain length causes a fall in inhibitory activity.

(2) The di-substituted compounds include substances with great inhibitory activity. This may seem surprising, as it is held that diamines have no affinity to amine oxidase. It can be shown, however, that this is not true for all diamines; we have recently found that the straight-chain diamines of the type $NH_2(CH_2)_nNH_2$ with 14, 16 and 18 $CH_2$ groups respectively are substrates of amine oxidase; there seems to be a maximum of affinity at a certain chain length (Blaschko & Duthie, unpublished).

The guanidine and isothiourea derivatives owe their inhibitory activity probably also to the presence of the amidine group. Here, too, the di-substituted derivatives are active as inhibitors.

There is no general parallelism of inhibitory action on amine oxidase and trypanocidal effect. For instance stilbamidine, which is highly active as a trypanocidal drug, is not a strong inhibitor. On the other hand, in the series of compounds which includes propamidine and pentamidine, there is a parallelism: the curves of both trypanocidal and inhibitory activities show maxima for the substances with 3 and 5 $CH_2$ groups. With the straight-chain diamidines we find the maximal inhibitory action in the $-(CH_2)_{12}-$ compound; King, Lourie & Yorke (1938) found the maximum trypanocidal activity in the compound with 11 $CH_2$ groups.

The demonstration of an inhibitory action of trypanocidal drugs on enzymes is of interest in connexion with possible toxic actions on the host, but it may also have some bearing on the action of these substances on the parasite. Little is known about the mechanism of their therapeutic action, but it seems possible that in the parasite the drugs may interfere with enzymic systems which are in some way chemically related to amine oxidase.

## SUMMARY

1. The inhibitory action of amidine derivatives on the amine oxidase activity of rabbit's liver has been examined.

2. Monoamidines, diamidines, mono- and di-guanidines and diisothiourea derivatives inhibit the enzyme.

3. With increasing length of the carbon chain there is at first an increase of activity but with a further lengthening of the chain there is a decrease of inhibitory action.

4. Stilbamidine is not a strong inhibitor of the enzyme, but propamidine and pentamidine inhibit very strongly. The pentamidine inhibition is at least partially reversible.

5. Marfanil and V 335 are substrates of amine oxidase, but the corresponding amidines (V 147 and V 187) have little affinity for the enzyme.

This work has been carried out while the authors held grants from the Medical Research Council.

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# The Kinetics of Low Temperature Acid Hydrolysis of Gramicidin and of some Related Dipeptides

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(*Received* 12 *June* 1945)

The work described in this paper was undertaken in order to throw light on the course of acid hydrolysis of gramicidin, and has especial significance in connexion with the analytical data already reported for a partial acid hydrolysate of gramicidin (Synge, 1944). The time course of evolution, during low temperature acid hydrolysis, has now been studied for amino groups, 'hydroxyamino' groups (i.e. free ethanolamine—Synge, 1945) and free amino-acids. Interpretation of the mode of breakdown of gramicidin has been assisted by parallel studies of the hydrolysis, under the same conditions, of a number of dipeptides embodying peptide bonds representative of those possibly present in gramicidin.

Data in the literature on the kinetics of acid hydrolysis of peptides, etc. are fragmentary and scattered (e.g. Abderhalden & Bahn, 1932, 1935; Abderhalden & Brockmann, 1927; Abderhalden & Köppel, 1927; Abderhalden & Mahn, 1927, 1928; Abderhalden & Sickel, 1927; Steiger, 1934), whereas extensive data on the kinetics of alkaline hydrolysis have been published, especially by Levene and Abderhalden and their respective collaborators. The peptides employed in the present experiments were mostly the same as those employed in a study of alkaline hydrolysis kinetics by Levene, Steiger & Rothen (1932). Comparison has therefore been possible of the effects of some amino-acid side-chains on the rate of hydrolysis of adjacent peptide bonds by acid and alkali; in this connexion the present observations have general significance in protein chemistry.

## EXPERIMENTAL

### *Materials*

*Synthetic dipeptides.* Glycyl-*dl*-alanine was prepared according to Fischer (1904), *dl*-alanylglycine according to Axhausen (1905) and glycyl-*l*-tryptophan according to Abderhalden & Kempe (1907). Other dipeptides employed were the preparations referred to by Synge (1944).

*Gramicidin.* Preparation R 3 (Synge, 1944) was employed.

### *Methods*

*Hydrolysis of synthetic dipeptides.* Unless otherwise stated, a 0·2 M solution of peptide in 10 N-HCl was diluted to 2 vol. with glacial acetic acid. The resulting mixture was kept in a glass-stoppered vessel in the incubator at 37° and 0·2 ml. portions were withdrawn at intervals for analysis.

The hydrolysis solutions remained colourless except for those containing tryptophan peptides. The mixture with *l*-leucyl-*l*-tryptophan rapidly darkened through purple to deep brown. Measured samples of the mixture with glycyl-*l*-tryptophan were kept in separate sealed evacuated tubes to prevent access of air; nevertheless, the solution progressively darkened to a dark green.

*Determination of extent of hydrolysis of synthetic peptides.* This was effected by the ninhydrin-$CO_2$ manometric microprocedure of Van Slyke, Dillon, MacFadyen & Hamilton (1941). The solution for analysis was evaporated to dryness in the reaction vessel over $H_2SO_4$ and soda-lime in a vacuum desiccator at room temperature overnight. The reaction was effected with 50 mg. ninhydrin and 50 mg. of the pH 4·7 citrate buffer in water at a volume of 2 ml.

'Blank' determinations on portions of the hydrolysis mixtures evaporated immediately after making up exceeded the reagent blank, and indicated that 3–10% hydrolysis occurred during the overnight evaporation. The results quoted below have been corrected by subtraction of the blank determined in this way for each peptide. This probably introduces an excessive correction of the readings in the later stages of the hydrolysis, but the effect is small.

Portions of each hydrolysis mixture were subjected to 'complete' hydrolysis by heating for 36 hr. in sealed evacuated tubes at 110–115° before the ninhydrin-$CO_2$ determination was done. The results lay within 5% of the theoretical for all the peptides except those containing tryptophan; glycyl-*l*-tryptophan gave 81% and *l*-leucyl-*l*-tryptophan 86% of the theoretical result for complete hydrolysis. However, in calculating the hydrolysis constants, the extent of hydrolysis of these peptides, as for the others, was expressed on the basis of the theoretical final value, since destruction of tryptophan residues is probably less serious at 37° than at 110–115°.

*Hydrolysis of gramicidin.* To 2·5 ml. portions of an accurately made up 2% (w/v) solution of gramicidin in glacial acetic acid were added 2·5 ml. 10 N-HCl and the resulting clear solutions were kept at 37° in sealed evacuated tubes. The appearance of the mixture as hydrolysis proceeds has been described by Synge (1944). At intervals, tubes were removed for analysis; their contents were transferred quantitatively with glacial acetic acid to a flask having a ground-glass joint connexion to the evacuation apparatus and evaporated to dryness *in vacuo* below 40°. (When water is employed for transference, the mixture froths uncontrollably, and precipitation occurs.) During the evaporation of the earlier hydrolysates there was separation of insoluble material, which sometimes took the form of a skin and impeded the evaporation; this decreased markedly with increasing time of hydrolysis, and after 20 days' hydrolysis no such phase separation occurred on evaporation. The evaporation residues were made to 2·5 ml. with glacial acetic acid, in which they were all completely soluble.

*Determination of amino-N of gramicidin hydrolysates.* This was done in duplicate on 0·25 ml. samples of the above acetic acid solutions. The solution was washed into the apparatus with a further 0·75 ml. glacial acetic acid (added in 2 portions) and then with 5 ml. water, after which the standard 4 min. Van Slyke manometric amino-N procedure was followed. The earlier hydrolysates gave precipitation when the water was added, and further precipitation and frothing on addition of the $NaNO_2$, but this did not occur with 20-day (or longer) hydrolysates. In all cases the reaction mixture turned yellow when the $NaNO_2$ was added.

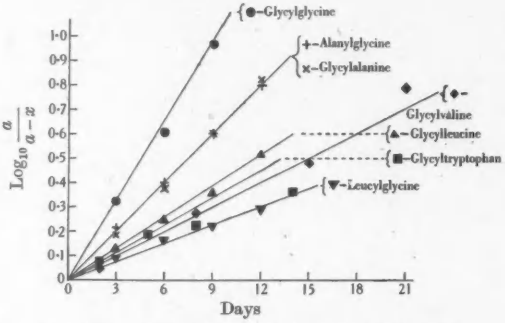


Fig. 1. Data on kinetics of hydrolysis of dipeptides. ($a$ = initial quantity of peptide; $x$ = quantity of peptide (same units) hydrolyzed after given time.)

*Determination of 'hydroxyamino'-N of gramicidin hydrolysates.* This was effected in Conway units by the adaptation of the periodate-$NH_3$ method of Van Slyke, Hiller & MacFadyen (1941) employed throughout for the work with the hydroxyamino constituent of gramicidin (Synge, 1945). Two 0·5 ml. samples of the acetic acid solution of evaporated hydrolysate were placed in the outer chamber of Conway dishes whose rims had been smeared with vaseline, and were allowed to evaporate overnight in a vacuum desiccator containing $H_2SO_4$ and soda-lime. One dish was employed for the $NH_3$ determination, and the other for the ($NH_3$ + 'hydroxyamino')-N determination. No free $NH_3$ was found at any stage of the hydrolysis. Since, on addition of the reagents to the dishes, much material, particularly in the early stages of hydrolysis, did not dissolve or was precipitated, it appeared that low values might be resulting from failure of reagents or $NH_3$ to diffuse through the lumps of insoluble material. Accordingly, the dishes were titrated as usual after 2 hr. equilibration, and were then re-closed for a further 22 hr., after which any further $NH_3$ which had reached the centre chamber was titrated. The results obtained after 2 and 24 hr. are recorded separately; the 2 hr. values have been corrected for the incomplete equilibrium absorption of $NH_3$ (95%), whereas the 24 hr. values (sum of 2 and 24 hr. titrations) are naturally not so corrected. All results are further corrected, a 90% yield of $NH_3$ from ethanolamine being assumed (cf. Synge, 1945).

A determination was also carried out on intact gramicidin which had been evaporated from acetic acid solution in the dishes. The gramicidin dried to a clear skin, which, on addition of the reagents, broke away from the dish and floated as flakes. No $NH_3$ or 'hydroxyamino'-N resulted.

*Determination of free amino-acids of gramicidin hydrolysate.* Duplicate 0·25 ml. portions of the acetic acid solution of gramicidin hydrolysate were each mixed with 50 mg. of the pH 4·7 citrate buffer, evaporated in the reaction vessel in a desiccator overnight, and subjected to the ninhydrin-$CO_2$ procedure.

## RESULTS

*Hydrolysis of synthetic peptides.* As expected, hydrolysis was found to follow the unimolecular law. Figs. 1 and 2 give the data, together with the

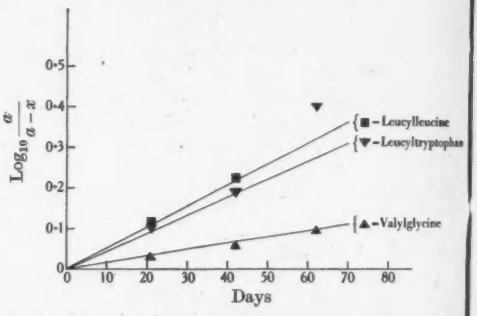


Fig. 2. Data on kinetics of hydrolysis of dipeptides. ($a$ = initial quantity of peptide; $x$ = quantity of peptide (same units) hydrolyzed after given time.)

best-fitting straight lines, from which the half-lives given in Table 1 have been deduced. It has seemed better, in view of the progressive decomposition of the tryptophan peptides, mentioned above, to draw their lines in agreement with the hydrolysis data from the earlier samples taken.

Table 1. *Hydrolysis of dipeptides by acid and alkali*

| Peptide | Half-life period (days) in a mixture of equal volumes of 10 N-HCl and glacial acetic acid at 37° | Relative velocity of hydrolysis (glycylglycine = 1) | |
|---|---|---|---|
| | | In acid (present study) | In alkali (Levene *et al.* 1932) |
| Glycylglycine | 2·8 | 1 | 1 |
| Glycyl-*dl*-alanine | 4·5 | 0·62 | 0·24 |
| *dl*-Alanylglycine | 4·5 | 0·62 | 0·21 |
| Glycyl-*dl*-leucine | 7·0 | 0·40 | 0·061 |
| Glycyl-*l*-tryptophan | 8·0 | 0·35 | — |
| Glycyl-*dl*-valine | 9·0 | 0·31 | 0·049 |
| *dl*-Leucylglycine | 12 | 0·23 | 0·048 |
| *l*-Alanyl-*l*-alanine | — | — | 0·043 |
| *dl*-Leucyl-*dl*-leucine 'A' | 58 | 0·048 | 0* |
| *l*-Leucyl-*l*-tryptophan | 68 | 0·041 | — |
| *dl*-Valylglycine | 190 | 0·015 | 0 |
| Glycyl-α-amino*iso*-butyric acid | — | — | 0 |

* *d*-Leucyl-*d*-leucine.

With glycyl-*dl*-alanine, the unimolecular mode of hydrolysis was further checked (i) by employing an initial concentration three times as great as usual, and (ii) by adding equimolar quantities of the hydrolysis products, glycine and *dl*-alanine, to the initial hydrolysis mixture. In both these experiments hydrolysis was unimolecular and the constant was the same as before.

*Hydrolysis of gramicidin.* In Table 2 the data on the hydrolysate of gramicidin are tabulated (*a*) as N in percentage of total N of the gramicidin and (*b*) as percentage of the theoretical figure for a peptide molecule at different stages of hydrolysis, as follows:

Expressed as percentage of total N of gramicidin, if $x$ = α-amino-N of free amino-acids, $(73{\cdot}3-x)$ the α-amino-N of amino-acid residues in peptide linkage (i.e. difference of free amino-acids before and after complete hydrolysis), $y$ the amino-N of the partial hydrolysate, and $z$ the 'hydroxyamino-N' (i.e. amino-N of free ethanolamine), then $y-x-z$ = free amino-N of peptides, and the average number of amino-acid residues per peptide molecule is $(73{\cdot}3-x)/(y-x-z)$. These values are shown in the

Table 2. *Course of hydrolysis of gramicidin in a mixture of equal volumes of* 10N-HCl *and glacial acetic acid at* 37°

((*a*) Values are N as percentage of total N of gramicidin.

(*b*) Values are as percentage of 'theoretical' values for a complete hydrolysate. These are taken to be: amino-N, 80% of total N of gramicidin; α-amino-N of amino-acids, 73·3% of total N of gramicidin; 'hydroxyamino'-N, 6·7% of total N of gramicidin.)

| Time of hydrolysis (days) | Amino-N | | α-Amino-N of free amino-acids | | 'Hydroxyamino'-N determined at | | | Average no. of amino-acid residues per molecule of peptide |
|---|---|---|---|---|---|---|---|---|
| | | | | | (*a*) | | (*b*) | |
| | (*a*) | (*b*) | (*a*) | (*b*) | 2 hr. | 24 hr. | 24 hr. | |
| 0 | 0* | 0* | (0) | (0) | 0 | 0 | 0 | — |
| 1 | 16 | 20 | 0·8 | 1 | 2·4 | 2·8 | 42 | 5·7 |
| 2 | 23 | 29 | 1·6 | 2 | 2·0 | 2·2 | 33 | 3·7 |
| 6 | 33 | 41 | 7·2 | 10 | 3·6 | 3·8 | 57 | 2·9 |
| 11 | 41 | 51 | 12 | 16 | 4·1 | 4·2 | 63 | 2·4 |
| 19¾ | 47 | 59 | 20 | 27 | 3·1 | 3·3 | 49 | 2·2 |
| 47 | 55 | 68 | 29 | 39 | 3·2 | 3·4 | 51 | 2·0 |

* Hotchkiss (1941).

complete hydrolysate, assuming the stoichiometric minimum molecule postulated by Gordon, Martin & Synge (1943*a*; cf. Synge, 1944, 1945). The actual

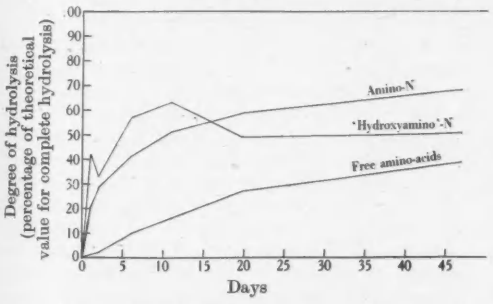


Fig. 3. Course of evolution of amino groups, 'hydroxyamino' groups and free amino-acids during low temperature acid hydrolysis of gramicidin.

values of amino-N and ninhydrin-$CO_2$ for a 'complete' hydrolysate approximate closely to these figures (Hotchkiss, 1941).

It has been possible to obtain an estimate of the number of residues other than ethanolamine per last column of Table 2. For similar calculations of peptide size during protein hydrolysis compare Gordon, Martin & Synge (1941), Van Slyke, Dillon *et al.* (1941) and Stein, Moore & Bergmann (1944). In the present instance, glycyl peptides are probably absent and the amino-N values are therefore unlikely to be too high.

The course of evolution of amino-N, α-amino-N of free amino-acids and 'hydroxyamino-N' as percentages of the theoretical maximum for each quantity is plotted in Fig. 3.

## DISCUSSION

When the synthetic dipeptides here studied are arranged in order of ease of hydrolysis by acid, it is seen (Table 1) that this sequence is the same (for those peptides common to both investigations) as that found for ease of hydrolysis by alkali (Levene *et al.* 1932). It should be noted, however, that when the velocity constants are expressed on a common scale (those for glycylglycine being taken as 1 both in acid and alkali) the differences are relatively greater for alkali than for acid. The influence of various alkyl side-chains on the hydrolyzability of an adjacent peptide bond is seen to be in the same

sense for acid as for alkaline hydrolysis. In view of the specially large inhibitory effects of the 'spreading' side-chains of valine and α-amino*iso*-butyric acid residues it seems reasonable to attribute the effects, at least in part, to steric limitation by the side-chains of the access of ions to the peptide bond.

Valylglycine is seen to be much the most resistant to acid hydrolysis of the dipeptides studied, and it is therefore easily understood why it was found in high yield, and substantially unaccompanied by free valine or glycine in a 10-day acid hydrolysate of gramicidin (Synge, 1944). Valylvaline is probably still more resistant; its occurrence after vigorous acid hydrolysis of gramicidin (Christensen, 1943, 1944) is thus readily explained.

It may be noted that the average number of amino-acid residues per peptide molecule in the 10-day gramicidin hydrolysate previously studied (Synge, 1944) is about $2\frac{1}{2}$ (Table 2).

The resistance to acid hydrolysis exhibited by peptides of the higher monoamino-acids with fatty side-chains, such as leucylleucine and valylvaline, suggests that if such amino-acids occurred directly linked to one another, corresponding dipeptides would have been found by Gordon, Martin & Synge (1943*b*) in the acid hydrolysates of gelatin studied by them and strengthens their suggestion that such linkages do not occur in gelatin.

It is reasonable, as a first approximation, to assume that the stability to hydrolysis of each bond in a long peptide chain will depend mainly on the nature of the amino-acid residues directly involved in it, in such a way that the relative stability of the various bonds will be correlated with that of the corresponding dipeptides. On this view, long peptide chains would, at least in respect of their monoamino monocarboxylic residues, be subject to initial attack at much the same points both by acid and alkali; a disappointing prospect, as already noted (Synge, 1944), for studies of protein structure by partial hydrolysis methods. However, although the same bonds in a long peptide chain are likely to be attacked initially, the subsequent effects of acid and alkali are likely to be different, since in acid ionic repulsion will protect the bond next to that broken and involving the residue whose amino group has been set free, while in alkali the corresponding bond involving the residue with a free carboxyl group will be protected.

This enhanced stability of dipeptides to acid and alkaline hydrolysis in comparison with larger peptides embodying the same amino-acid residues was postulated by Gordon *et al.* (1941; cf. Synge, 1943) on the basis of physico-chemical theory and of impressions gained from a wide survey of the literature. It is now possible to adduce some more direct evidence in respect of the reality of the effect in acid hydrolysis:

(1) The observations of Stein *et al.* (1944) on the low temperature acid hydrolysis of silk show that consistently fewer free amino-acids and more dipeptides are present in the hydrolysate than would be the case if the splitting of peptide bonds were random, on which assumption, when $1/n$ of the bonds in an original long-chain peptide had been split, $1/n^2$ of the amino-acid residues present would be free. (However, these effects were not so marked with the proteins studied by Gordon *et al.* (1941).)

(2) The present study with gramicidin shows that the absolute rate of hydrolysis of gramicidin in its initial stages greatly exceeds the rate of hydrolysis of dipeptides corresponding to bonds possibly present, and further that the number of free amino-acids at any single stage is less than the 'random' expectation. Christensen (private communication)* has also noticed the latter phenomenon with low temperature acid hydrolysis of gramicidin, and states that it is less marked with hydrolysis at higher temperatures. This diminution of the effect on raising the temperature is to be expected with the ionic repulsion mechanism already suggested.

(3) Christensen (1943, 1944) has found the peptide bond in valylvaline much more resistant to acid hydrolysis than the same bond in benzoylvalylvaline.

In comparing the course of hydrolysis of silk (Stein *et al.* 1944) with that of gramicidin, it is interesting to note that peptides higher than dipeptides persist to a later stage of the hydrolysis of gramicidin than of silk. This may perhaps be correlated with the different amino-acid composition of the two substances. Kinetic interpretation of the data of Stein *et al.* is rendered difficult by the fact that they employed only a slight excess of acid for hydrolysis.

For studies of the structure of peptides and proteins by the recognition of partial hydrolysis products, it would in general be desirable to employ a hydrolytic agent that did not discriminate in its attack on different peptide bonds, were it not for technical difficulties of separating the multitude of representative compounds, thus produced. A good alternative would be to employ in separate studies a number of more selective hydrolytic agents, each preferentially attacking different bonds in the compound under investigation. Until the synthetic powers of proteolytic enzymes are better understood, acid and alkali are the main hydrolytic agents of practical use, and it is hoped that the considerations presented here may be of help towards using them with good effect. The disadvantage that both may tend to attack initially the same bonds may turn out to be offset by their subsequent preferential attack on different bonds; further, the ten-

* (*Note added in proof.*) See Christensen, H. N. & Heysted, D. M., *J. biol. Chem.* (1945), **158**, 593.

dency of any bond in the original molecule to acquire increased stability to acid or alkali once it is embodied in a dipeptide molecule should be definitely advantageous.

## SUMMARY

1. The hydrolysis velocity constants for a number of dipeptides in acid at 37° have been determined, and are compared with the data of Levene *et al.* (1932) for similar dipeptides in alkali.

2. The course of hydrolysis of gramicidin by acid under the same conditions has been followed by determination of the evolution of amino groups, free amino-acids and 'hydroxyamino' groups.

3. The nature of the process of hydrolysis by acid of long peptide chains is discussed in relation to the available data.

I wish to thank Mr W. Wolff for his technical assistance.

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# The Hydroxyamino Component of Gramicidin Hydrolysates

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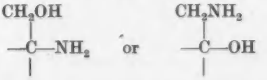
(*Received* 12 *June* 1945)

The presence of a compound in acid hydrolysates of gramicidin, containing the groupings



was inferred by Hotchkiss (1941) from the fact that periodate liberates formaldehyde and $NH_3$ from such hydrolysates (cf. Christensen, Edwards & Piersma, 1941; Synge, 1944*a*). Hotchkiss further showed that the material responsible for these reactions with periodate was not an α-amino-acid, since the α-amino-acid content of hydrolysates (determined by the ninhydrin-$CO_2$ procedure) was unaffected by treating them with periodate. Serine was not therefore responsible for the reaction (cf. Gordon, Martin & Synge, 1943*b*). Hotchkiss rejected the idea that the material might be *iso*serine or hydroxylysine, and noted its tendency to decompose, with liberation of $NH_3$, during isolative manipulations. Failure to isolate even small amounts of aldehydo-acid as acidic hydrazone after oxidation with periodate led Hotchkiss later (1944) to doubt that the hydroxyamino component was a carboxylic acid. As a result of chromatographic experiments with acetylated hydrolysates of gramicidin, the same doubt had occurred to the present author, and experiments were undertaken to test for the presence in gramicidin hydrolysates of volatile amino-alcohols.

*Occurrence of ethanolamine in gramicidin hydrolysates*

It was found that on azeotropic distillation with toluene of an acid hydrolysate of gramicidin which had been rendered alkaline with excess of $K_2CO_3$, ethanolamine (2-aminoethanol-1) was recovered in the distillate; control experiments showed that this could not be derived as a simple artefact from the known amino-acid constituents of gramicidin. A short note of this result has already been published (Synge, 1944*b*). The ethanolamine has been identified by: (1) its elementary composition; (2) its content of basic groups and amino-N; (3) the yield of $NH_3$, acetaldehyde (none) and formaldehyde (2 molecules) obtained on treatment with periodate; (4) its volatility, optical inactivity, and ionophoretic behaviour; (5) the preparation from it of a chloroaurate which has been found crystallographically identical with authentic ethanolamine chloroaurate.

Since the procedure first employed for isolating the ethanolamine was rather violent, it was desirable to isolate it by a milder treatment, not involving high temperatures or rendering the hydrolysate alkaline. This was achieved by a new ionophoretic technique devised by Dr A. J. P. Martin and his colleagues (Consden, Gordon & Martin, 1946; see Martin & Synge, 1945). By this technique ethanolamine was isolated from acid-hydrolysates of gramicidin after simple neutralization to pH 6.

The yields of ethanolamine obtained by both the distillation and ionophoretic procedures do not suggest the presence of other hydroxyamino compounds, there being sufficient ethanolamine present to account for all of the $NH_3$ liberated by periodate treatment of the same unfractionated acid hydrolysates of gramicidin. No $CO_2$ was liberated during acid hydrolysis of gramicidin.

Ethanolamine was also isolated ionophoretically from partial hydrolysates of gramicidin obtained by the action of acid for 2 days at 37°. At the same time, a basic fraction, having a lower migration rate and yielding ethanolamine and amino-acids on further acid hydrolysis, was isolated. The nature of this fraction is discussed at greater length below.

The above data may be taken to indicate that the ethanolamine is not derived by decarboxylation of an amino-acid, and that ethanolamine residues possibly occur as such in the undegraded structure of gramicidin.

On heating with ninhydrin, ethanolamine gives a colour considerably weaker than that due to an equivalent of amino-acid. More acute observation of paper strip chromatograms with gramicidin hydrolysates (Synge, 1944*a*) would have led to its recognition as a faint band moving more slowly than the glycine band with butanol-$H_2O$. The chromatographic experiments of Gordon *et al.* (1943*b*) could not, however, have been expected to detect its neutral *N*-acetyl derivative.

*Quantitative determination of ethanolamine in gramicidin*

An estimate of the number of ethanolamine residues in the stoichiometric minimum unit of gramicidin has been complicated somewhat by the incomplete yield of $NH_3$ obtained on treating ethanolamine with periodate, and more seriously by the instability of ethanolamine under the conditions employed for hydrolyzing gramicidin. The results of applying the periodate-$NH_3$ reaction to ethanolamine obtained by other workers are given in the experimental section; the method employed in the present work gave sufficiently constant recoveries (90%) to permit a correction factor to be applied with some confidence to all determinations.

When gramicidin was hydrolyzed for 24 hr. at 110–115° in a 1 : 1 mixture of glacial acetic acid and 10N-HCl, the ethanolamine-N in the hydrolysate was found to be 3·0–3·5% of the total-N of gramicidin. Higher values (4·7–5·1%) were found with hydrolysates prepared at a slightly lower temperature and containing less acetic acid (Hotchkiss, 1941; Synge, 1944*a* and present work). Control experiments showed that ethanolamine heated by itself in these acid solutions is considerably destroyed, and that in the presence of a mixture of the known amino-acid constituents of gramicidin the destruction is still greater. As with hydrolysates of gramicidin itself, higher yields were obtained from the mixtures subjected to the lower temperatures and lower acetic acid concentrations. When the ethanolamine figures for gramicidin hydrolysates are corrected by the recoveries obtained in these control experiments, they are found to comprise, as ethanolamine-N, 5·5–6·0% of the total-N of gramicidin. The figure 6·7% would represent two residues of ethanolamine in the postulated stoichiometric minimum unit of gramicidin, and it is not unreasonable to suppose that in fact two such residues are present, and that the destruction of ethanolamine residues during their liberation from chemical linkage is slightly greater than the destruction observed with free ethanolamine.

The observations of Synge (1945) on the course of liberation of ethanolamine in low temperature hydrolysis of gramicidin give direct evidence of destruction, which is as great in the 1 : 1 acetic acid-10N-HCl mixture at 37° as at 110–115°. It is suggested that in the high concentrations of acetic acid, there may be a significant equilibrium Fischer-Speyer esterification to *O*-acetylethanolamine, which might be expected, under the alkaline conditions of the periodate-$NH_3$ determination, not to saponify

simply, but to rearrange to *N*-acetylethanolamine (Bergmann & Brand, 1923; Bergmann, Brand & Weinmann, 1923) and thus escape reaction. At the higher temperatures, destruction of ethanolamine probably occurs in other ways, since liberation of $NH_3$ is observed. The breakdown of serine in hot acid provides known analogies, and the same reaction which produces pyruvic acid from serine (Abderhalden & Broich, 1933; Damodaran & Ramachandran, 1941) would explain the formation from ethanolamine of the small quantities of acetaldehyde noticed by Hotchkiss (1944) in a hydrolysate of gramicidin.

The ethanolamine yield from different preparations of gramicidin hydrolyzed in the same way appeared to be the same. The small quantities of fatty acid and P present in gramicidin, as well as varying in quantity from one preparation to another, were in every case inadequate to account for the ethanolamine being derived from contaminating phospholipid (cephalin) (cf. Table 2).

### *Mode of incorporation of ethanolamine residues in gramicidin*

The evidence now presented, taken with the conclusions of Gordon *et al.* (1943*b*) and Synge (1944*a*) leads to the postulate that the stoichiometric minimum unit of gramicidin embodies residues of amino-acids, etc., as follows, which account completely for the N of gramicidin: 6 tryptophan (*l*); 6 leucine (*d*); 5 valine (*d* and *l*); 3 alanine (*l*); 2 glycine; 2 ethanolamine.

If the ethanolamine residues are involved in peptide linkage through the amino group, and have the hydroxyl group free, the hydroxyl groups of gramicidin (Hotchkiss, 1941) are then adequately accounted for.

Such a stoichiometric minimum unit, independent of its degree of condensation, hydration, etc. would have 144 C atoms per 30 N atoms. The data of Hotchkiss & Dubos (1941) on the elementary composition of gramicidin give the somewhat higher ratio of 148 C per 30 N, suggesting that some non-nitrogenous carbon-containing residue may still remain to be found. The possibility of firmly bound acetone of crystallization has not been excluded; acetone of crystallization was noticed in preparing acetyl-*dl*-leucylglycine (Gordon, Martin & Synge, 1943*a*).

There are more serious difficulties involved in regarding gramicidin as being formed simply by peptide condensation of the above residues. Unless abnormal linkages are assumed, the resulting unit would have a free basic or amino group, and there is evidence that gramicidin does not possess such groups (Hotchkiss, 1941). The fact that it forms a crystalline compound with flavianic acid (Tishler, Stokes, Trenner & Conn, 1941) is scarcely significant, since such acids are well known to form stoichiometric 'molecular compounds' with neutral substances such as aromatic hydrocarbons.

The 'extra' carbon of gramicidin as well as its neutral character would be readily explained were carboxylic acid residues also present. Hotchkiss (1941) states that carboxylic acids (volatile or non-volatile, ether-extractable) are absent from gramicidin hydrolysates. In the present study likewise, exhaustive extraction with ether, adequate to isolate oxalic acid, failed to reveal the presence of such an acid.

A less plausible explanation of the neutral character of gramicidin could be the presence of carbonic acid residues (ureido groups) linking two amino-acid residues thus:

$$—CHR.NH.CO.NH.CHR'—.$$

Such groupings would not, on acid hydrolysis, necessarily liberate $CO_2$ (cf. Corwin & Damerel, 1943; Gordon, Martin & Synge, 1943*c*) but might be expected to give, on mild acid hydrolysis, nitrogenous products bearing a net negative charge at pH 6 (cf. Locquin & Cerchez, 1931; Cerchez, 1931). However, in the ionophoretic experiment with a 2-day partial hydrolysate described below, no significant quantity of N was found to migrate towards the anode (see Table 5). The possibility of both the glycine and ethanolamine of the hydrolysate arising by Cannizzaro rearrangement of aminoacetaldehyde residues should not be ignored (cf. Trier, 1911).

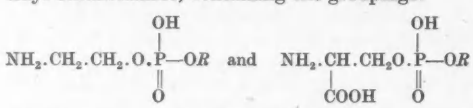
A satisfactory account of the over-all properties of gramicidin in terms of its constituent residues must await further work. Leaving this problem on one side, it seemed possible to investigate the mode of linkage of ethanolamine residues in gramicidin by examining the basic fractions obtained by ionophoresis from a 2-day low-temperature acid hydrolysate. Here it might be expected that a proportion of ethanolamine residues would be found bound as relatively simple 'peptides'.

The analytical data presented in the experimental section for such a fraction show: (i) that the fraction probably consists of 'peptides' involving ethanolamine, from which ethanolamine can be liberated on acid hydrolysis; (ii) that significant quantities of tryptophan are absent from it; (iii) that the total quantity of ethanolamine found in the fraction corresponds to only $\frac{1}{4}$–$\frac{1}{3}$ of the ethanolamine that is free in the original hydrolysate. This low yield of bound ethanolamine can be ascribed to the fact that ethanolamine appears to be set free from gramicidin in acid hydrolysis faster than the average peptide bond is broken (Synge, 1945, Fig. 3). A hydrolysate of the 'ethanolamine peptide' fraction was examined by a butanol-$H_2O$ paper strip chromatogram (Consden, Gordon & Martin, 1944; cf. Synge, 1944*a*) and revealed the presence of glycine, alanine,

valine and leucine as well as of ethanolamine. Two-dimensional chromatograms (Consden *et al.* 1944) with the intact 'ethanolamine peptide' fraction showed at least four major components in it, none of which, as control experiments showed, was the simple 'peptide', *N*-alanylethanolamine or *N*-leucylethanolamine. Thus this basic peptide mixture is both low in yield and complex in nature. It may contain artefacts formed by esterification of ethanolamine —OH groups with amino-acids and peptides (see above).

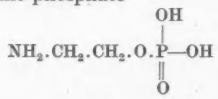
Gramicidin appears to be the first case described of the natural occurrence of ethanolamine (2-amino-ethanol-1) linked through its amino group. The next higher homologue (2-amino-propanol-1) is found linked in this way in ergometrine (for bibliography see Guggenheim, 1940, pp. 119, 158). This is by no means the only point of chemical similarity between the ergot 'alkaloids' and the 'polypeptides' derived from *Bacillus* spp.

A possible significance of the finding of ethanolamine residues in gramicidin for the mechanism of its antibacterial activity should be mentioned. Modern views on the chemical mechanism of antibacterial action lay special stress on the inhibition of enzyme systems by substances of structure related to that of normal substrates, coenzymes, etc. It may prove not merely coincidental that gramicidin (1) possibly contains, according to data presented here, ethanolamine residues linked thus: $R.CO.NH.CH_2.CH_2OH$; (2) interferes with bacterial growth and anabolism and, more specifically, with the metabolism of phosphate (Hotchkiss, 1944); (3) is inhibited in these activities by the cephalins *O*-phosphatidyl-serine and *O*-phosphatidyl-ethanolamine, containing the groupings:



(for references see Hotchkiss (1944))

In this connexion the isolation by Outhouse (1937) of ethanolamine phosphate



from cancerous (growing) tissue may have some significance.

## EXPERIMENTAL

### *Materials*

Three specimens of *gramicidin*, W2, R3 and RM, were employed in the present study. The origin and preparation of W2 and R3 was described by Synge (1944*a*). RM was the material left in the mother liquors during the preparation there described of R3 from R1.

The authentic ethanolamine employed for identification and control experiments was a commercial sample kindly given by Dr F. A. Robinson for which amino-N (Van Slyke), basic groups (titration with HCl) and the yield of formaldehyde on periodate oxidation bore the theoretical ratios to one another.

### *Analytical methods*

$NH_3$ *liberated by periodate.* These determinations were carried out in Conway units, employing the reagents recommended by Van Slyke, Hiller & MacFadyen (1941). The method has been described by Gordon, Martin & Synge (1943*d*) (cf. Ramsay & Stewart, 1941; Synge, 1944*a*). In the present work, the dishes were equilibrated for 2 hr. at room temperature without shaking. Allowing the usual correction for incomplete equilibrium absorption of $NH_3$ in the boric acid, 100% recovery of $NH_3$ from serine was found as before. On the same basis ethanolamine gave 87–93% recoveries of $NH_3$ consistently, and the figure of 90% recovery has been employed as a further correction of ethanolamine figures throughout this work. $NH_3$ blanks were run in parallel with all periodate-$NH_3$ determinations and periodate-$NH_3$ figures quoted refer to the difference of the pair of determinations. Van Slyke *et al.* (1941) noted yields of 85% of $NH_3$ from ethanolamine by their aeration procedure. Artom (1945), by a steam distillation procedure, has obtained theoretical yields.

*Acetaldehyde and formaldehyde liberated by periodate.* These were both determined in the same oxidation mixture by Mr M. W. Rees, Cambridge, by an unpublished modification of the methods of Shinn & Nicolet (1941) and Nicolet & Shinn (1941). The method gives theoretical yields of the aldehydes from threonine and serine, and control experiments with ethanolamine gave no acetaldehyde and the theoretical yield (2 molecules) of formaldehyde (cf. Jones, 1944).

*Other estimations.* Tryptophan: the colorimetric procedure of May & Rose (1922) was employed; P was determined colorimetrically after incineration with $H_2SO_4$ and perhydrol by Dr M. G. Macfarlane; C and H were determined by Dr G. Weiler, Oxford.

### *Isolation of ethanolamine by azeotropic distillation with toluene*

Isolation of ethanolamine from biological material (phospholipid hydrolysates, etc.) has usually been accomplished by the use of precipitants or insoluble derivatives. Blix (1940) took advantage of its volatility, and employed a distillation method. Artom (1945) has employed a chromatographic method. For the present work distillation seemed advantageous, and use was made of toluene as a carrier solvent, since it forms positively azeotropic mixtures with ethanolamine as well as with water, and thus hastens the removal of ethanolamine from the non-volatile residue.

The toluene used was first washed with acid and distilled from soda-lime through a fractionating column at atmospheric pressure. An ordinary continuous liquid extraction apparatus (such as that illustrated (*b*-4632) in the catalogue of A. Gallenkamp and Co. Ltd.) was employed for the ethanolamine distillation. The HCl hydrolysate from which it was desired to isolate ethanolamine was first evaporated to dryness *in vacuo* in the flask of the apparatus; the residue was dissolved in 1–2 ml. of saturated $K_2CO_3$ solution in the

flask, which was then half filled with toluene. The apparatus was then assembled; the distilling flask was placed in an oil-bath which was heated so that the toluene boiled briskly. This caused the condensed distillate to bubble through the water at the bottom of the extraction tube before returning to the distilling flask. Ethanolamine was extracted by the water from the distillate. Soon after distillation had begun, the aqueous solution in the distilling flask lost water, and became a hard white crust on the walls of the flask. Distillation was continued for 3 hr. At the end of this time the water layer was collected from the extraction tube, which was rinsed out three times with distilled water. The resulting combined aqueous solution was then evaporated to about one-third its original volume below 40° *in vacuo*. In this way any $NH_3$ present was removed without significant loss of ethanolamine. Ethanolamine was determined in this solution, or in a suitable sample of it, by titration with 0·01 N-HCl, employing the indicator of Ma & Zuazaga (1942). Periodate-$NH_3$ determinations on the same solutions gave concordant results.

Control experiments showed that mixtures of the known amino-acids of gramicidin subjected to the same acid hydrolysis procedure as gramicidin, and to this isolation procedure, yielded no basic material; when ethanolamine was added to the mixture immediately before the distillation, 90% of it was recovered.

### *Characterization of ethanolamine isolated from hydrolysates of gramicidin*

Gramicidin preparation RM (212 mg.) was hydrolyzed in a sealed evacuated tube, with 2 ml. of glacial acetic acid and 2 ml. 10 N-HCl, for 24 hr. at 110–115°. The hydrolysate was treated as above for the isolation of ethanolamine, and samples of the resulting basic aqueous solution, free from $NH_3$, were employed for the following determinations: (1) titration of basic groups with HCl (in duplicate as above); (2) periodate-$NH_3$ determination; (3) formaldehyde and acetaldehyde evolved on oxidation with periodate; (4) N (Kjeldahl); (5) amino-N (Van Slyke: 4 min.).

Analysis (1) was done in the outer compartment of Conway dishes. The resulting solutions were then evaporated *in situ*, and the residues were employed for analysis (2). Control experiments showed that the presence of indicator did not interfere with the periodate-$NH_3$ reaction. The results of the analysis are expressed, in Table 1, on the basis of the basic group titration.

Table 1. *Analyses on solution of ethanolamine isolated from gramicidin*

| Analysis for | Result (units per basic equiv. as titrated with acid) |
|---|---|
| Hydroxyamino-N (periodate-$NH_3$) (atoms) | 1·03 |
| Acetaldehyde liberated by periodate (molecules) | 0·008 |
| Formaldehyde liberated by periodate (molecules) | 2·30 |
| N (Kjeldahl) (atoms) | 1·09 |
| Amino-N (atoms) | 0·98 |

On the basis of the previous titrations of samples with HCl, the amount of HCl required for neutralization was added to the rest of the solution, and it was evaporated to dryness *in vacuo* below 40°. The residue (dried over $H_2SO_4$—soda-lime *in vacuo* at room temperature) took the form of deliquescent needle-like crystals arranged in rosettes on the bottom of the flask. Its weight was 113 g./equiv. of HCl employed for neutralization. It had $[\alpha]_D^{16.5°}$, 0° ± 2° (water, $l = 0.5$, $c = 0.9$). Found: C, 26·0; H, 9·6% (calc. for ethanolamine hydrochloride, $C_2H_7ON.HCl$: equiv. wt. 97·5; C, 24·6; H, 8·2%).

The high periodate-formaldehyde, equivalent weight and C figures suggest that glycerol (derived from fatty material contaminating specimen RM—see below) may have been present. Ethanolamine samples isolated from specimen W2 which contained less fatty contamination (see Table 2) gave formaldehyde: —$NH_2$ ratios within 2% of the theoretical. There may also be some slight contamination by non-basic nitrogenous material, perhaps derived from indole.

From an ethanolamine HCl sample isolated in the same way from a hydrolysate of gramicidin R3: (1) by periodate oxidation and treatment with dimedone according to Nicolet & Shinn (1941) formaldehyde was isolated and identified as its crystalline dimedone derivative (m.p. 187° uncorr., not depressed on admixture of authentic material having m.p. 189°); (2) a 'gold salt' was prepared by treating with three parts by weight of commercial 'brown gold chloride' in concentrated aqueous solution in the presence of excess HCl. As with authentic ethanolamine, long yellow needles crystallized out readily (cf. Trier, 1911). The crystals, together with a sample of authentic ethanolamine chloroaurate, were submitted to Dr D. Crowfoot and Mr G. M. J. Schmidt, Department of Chemical Crystallography, Oxford, who report:

'The two specimens were of somewhat different appearance; the product from natural sources was in the form of thin very long needles, whereas the synthetic sample consisted of thick prisms. Optical and X-ray examination, however, proved them to be identical.

'The crystals are monoclinic needles or prisms elongated along [001], and bounded by (010), (110) and terminal faces not indexed. Their birefringence is very high; their most readily determined optical characteristic is the extinction direction which lies at an angle of 24° to [001] in (010). The unit cell dimensions determined by X-ray methods are $a = 7{\cdot}50$, $b = 14{\cdot}40$, $c = 4{\cdot}42$ A, $\beta = 112{\cdot}5°$, space group $P2_1$. The crystals agree in morphology and optics with the microphotograph and descriptions given by Knorr (1897) and by Grubenmann in Trier (1911).'

### *Yields of ethanolamine isolated from different gramicidin preparations*

The yields of ethanolamine, determined by titration after azeotropic distillation with toluene as above, are shown in Table 2 for the three gramicidin specimens studied. Hydrolysis was effected in a mixture of equal volumes of glacial acetic acid and 10 N-HCl for 24 hr. at 110–115° as above. The content of P of the gramicidin specimens and the fatty acid content of the hydrolysates are also given, expressed in units equivalent to the ethanolamine figures. The fatty acid was determined by extraction of aqueous suspensions of evaporated hydrolysates with three successive half-volume portions of B.P. chloroform. The combined chloroform extracts were evaporated and stored over soda-lime

in a vacuum desiccator overnight, dissolved in ethanol, and titrated, using phenolphthalein as indicator, with 0·01 N-aqueous $Ba(OH)_2$. Precipitation, presumably of 'barium soap', occurred during the titrations. The acid equivalent weights of the chloroform extracts lay in the range 200–350.

Table 2. *Ethanolamine, P and fatty acid contents of gramicidin specimens*

| | Yield (% of total N of gramicidin) | | |
|---|---|---|---|
| Specimen ... | R3 | RM | W2 |
| Ethanolamine N | 3·2 | 3·3 | 3·2 |
| P as N (1 atom P = 1 atom N) | 0·7 | 0·2 | 0·03 |
| Fatty acid as N (1 equiv. acid = 1 atom N) | 0·8 | 2·0 | 0·4 |

### *Recovery of ethanolamine after heating in* HCl-*acetic acid mixture*

Ethanolamine was treated (by itself and in the presence, in the appropriate proportions, of the amino-acid constituents of gramicidin) under the different conditions that have been employed for hydrolyzing gramicidin, viz. in sealed evacuated tubes (A) in excess of 1 vol. acetic acid +4 vol. 5 N-HCl for 48 hr. at 100° (cf. Synge, 1944*a*); (B) in excess of 1 vol. acetic acid: 1 vol. 10 N-HCl for 24 hr. at 110–115° (as in isolative experiments described above).

After evaporation of the acid *in vacuo*, the periodate-$NH_3$ reaction was carried out on the residue. The results are shown in Table 3. When ethanolamine was added to the amino-acid mixture *after* it had been heated under conditions (B), 102% recovery was given by the analysis.

Table 3. *Recoveries of ethanolamine determined by periodate-*$NH_3$ *reaction*

| | Recovery (%) | |
|---|---|---|
| Hydrolysis conditions | (A) | (B) |
| Without amino-acids | 97 | 76 |
| With amino-acids | 82 | 57 |

### *Determination of ethanolamine in gramicidin hydrolysate by periodate-*$NH_3$ *reaction*

This was done on gramicidin hydrolysates exactly as with the made-up mixtures above. Specimen R 3 hydrolyzed under conditions (A) gave ethanolamine-N 4·7% of its total N. Corresponding figures were obtained, after similar hydrolytic treatment, by Hotchkiss (1941): 5·1% corr.; and Synge (1944*a*): 4·8%, 5·1% corr. Under hydrolytic conditions (B), with all three specimens of gramicidin figures lying in the range 3·3–3·5% were obtained, in agreement with the isolative data reported in Table 2. The data for the same determination on hydrolysates of gramicidin prepared by acting with acid at 37° for various times are given by Synge (1945).

### *Carbon dioxide evolved in hydrolysis of gramicidin*

This was determined according to Van Slyke & Folch (1940), after connecting the sealed tube in which the hydrolysis had been conducted to the Van Slyke-Neill manometric apparatus (cf. Gordon *et al.* 1943*c*). A blank determination with 2 ml. glacial acetic acid and 2 ml. 10 N-HCl heated for 24 hr. at 110–115° gave 0·3 mg. $CO_2$, while 212 mg. of gramicidin RM, heated under the same conditions in the same mixture, gave only 0·2 mg. $CO_2$. Probably the neutralization of some of the HCl by amino-acids liberated decreased the destruction of acetic acid.

### *Ether-extractable acid in gramicidin hydrolysates*

A hydrolysate of 54 mg. gramicidin RM made under conditions (A) above, was evaporated to dryness *in vacuo*, dissolved in water, and extracted with chloroform as in the fatty acid determinations above. The aqueous layer was then transferred to the extraction chamber of a continuous liquid extractor, and extracted overnight with ether that had been purified by successive washing with $NaHSO_3$ and NaOH solutions and distilled wet through a fractionating column. The ether extract, after concentration *in vacuo* and keeping overnight in a vacuum desiccator over soda-lime, required only 0·08 ml. 0·01 N-$Ba(OH)_2$ for neutralization to phenolphthalein. A corresponding mixture of the known components of gramicidin (including ethanolamine), on treatment in the same way, yielded an extract requiring 0·12 ml. for neutralization. When 1·3 mg. of succinic acid were added to this mixture after heating it with acid but before the chloroform extraction, 98% of the succinic acid was titrated in the ether extract. It may be inferred from the data in *International Critical Tables* that at least one-third of any oxalic acid present would have been recovered by this procedure.

### *Ionophoretic isolation of ethanolamine from gramicidin hydrolysates*

The methods employed for this purpose have been developed by Drs R. Consden, A. H. Gordon & A. J. P. Martin (1946), who kindly made them available for the present work. The ionophoresis was carried out in a long slab of dilute silica jelly strengthened with paper pulp and embodying a 0·2 M-acetic acid-sodium acetate buffer solution of pH 6. The electrodes applying the potential to the slab were kept continuously washed with a stream of the same buffer solution, to prevent pH changes and carry away electrode reaction products. The solution to be investigated was inlaid in the slab ($22 \times 1\frac{1}{2}$ in., and less than $\frac{1}{4}$ in. deep) in a gutter cut across it about one-third of the distance from anode to cathode. A glass lid lay on the slab.

The factors determining migration and separation of substances in the apparatus cannot be discussed fully here. It must be said that in addition to the net charge and ionic mobilities of the various substances, electroendosmosis of fluid in the pores of the gel and adsorption on the micelles of the gel are two important factors determining migration rates. The electroendosmosis was demonstrated by the migration towards the cathode of monoamino-monocarboxy-acids having no net charge at pH 6; the adsorption by the considerable increase in the migration rates under the same applied voltage of ethanolamine, lysine, etc. on substituting sodium acetate with ammonium acetate buffer. (Dr Martin interprets this effect as due to competitive elution by ammonium ions of the amino compounds adsorbed on the silica micelles.)

In spite of difficulty in interpreting theoretically the effects observed, the procedure has proved useful for effecting preparatively the isolations required in the present work, and the empirically determined migration rates have provided further evidence of the identity of the hydroxyamino component of the hydrolysates with ethanolamine.

### *Ionophoretic recognition of ethanolamine in complete hydrolysate of gramicidin*

Gramicidin RM (20 mg.) was hydrolyzed by procedure (B) above. After evaporation of excess acid the residue was dissolved in water, the pH adjusted to 6 by addition of NaOH, and 1 mg. each of *dl*-lysine and *l*-aspartic acid added as 'markers'. The resulting mixture was subjected to ionophoresis, with the apparatus running for 12 hr. with 130 V. across the electrodes. The resulting current was about 150 ma. At the end of this time a filter-paper print from the jelly was coloured by spraying with 1% ninhydrin solution in butanol containing 20% (v/v) of acetic acid, followed by careful heating at 70–80°. This revealed four bands, as shown in Table 4.

Table 4. *Results of ionophoresis of gramicidin hydrolysate*

| Band No. | Band corresponding to | Migration of centre of band (cm.) | Migration towards | % of hydroxyamino-N of gramicidin hydrolysate recovered in corresponding fraction |
|---|---|---|---|---|
| 1 | Ethanolamine | 20 | Cathode | 76 |
| 2 | Lysine | 13·5 | Cathode | 5 |
| 3 | Monoamino-acids | 5·5 | Cathode | 1 |
| 4 | Aspartic acid | 8 | Anode | 1 |

The ethanolamine band on the prints was less strongly coloured than the amino-acid bands; its colour characteristically developed to a maximum more slowly. Control experiments with known mixtures showed that authentic ethanolamine migrated under these conditions at the same rate relative to lysine and the monoamino-monocarboxylic acids. Only two bands (corresponding to ethanolamine and to monoamino-monocarboxylic acids) could be detected in 'complete' hydrolysates of gramicidin examined ionophoretically without added 'markers'. When the fractions were worked up (by drying the jelly, and extracting the dry material with 1% (v/v) acetic acid solution), the hydroxyamino-N (periodate-$NH_3$) was determined on each. The results expressed in Table 4 show clearly that ethanolamine is responsible for the $NH_3$ liberated by periodate from gramicidin hydrolysates.

### *Ionophoretic analysis of a partial hydrolysate of gramicidin*

The data of Synge (1945, Fig. 3) indicated that simple peptides embodying ethanolamine residues might best be sought in a 2-day partial hydrolysate of gramicidin at 37°. Accordingly, after pilot experiments, 80 mg. of gramicidin RM were subjected to hydrolysis under these conditions, and the hydrolysate was evaporated to dryness below 40°. Addition of water to the residue caused separation of much friable, partly crystalline insoluble matter, and on adjusting this suspension to pH 6 with NaOH further precipitation occurred. The resulting suspension was transferred fairly completely to the ionophoresis apparatus, and subjected to the same treatment as above. On 'printing' and colouring with ninhydrin, three bands were observed: (1) corresponding to ethanolamine, (3) to the compounds bearing no net charge at pH 6, and (2) a weak band separate from (3) having its centre about one-third of the distance from (1) to (3). Only band (3) showed a blue colour on spraying a print with 0·1% *p*-dimethylaminobenzaldehyde in 10N-HCl without subsequent heating. This indicated that bands (1) and (2) were substantially free from tryptophan. The jelly of the ionophoresis apparatus was accordingly cut into fractions, as shown in Table 5, which were worked up and analyzed with the results there shown.

Fraction B (containing water-insoluble material) was extracted, after drying, with glacial acetic acid, and hydrolyzed by procedure (A) above before analysis. Hydrolysis of the other fractions was effected on samples in 6N-HCl for 24 hr. at 100°.

It is seen that the quantities available for analysis were near the lower limits of the analytical methods, which were therefore not as accurate as could be wished. However, the analyses justify the main conclusions drawn in the discussion part of this paper. The relatively large quantity of ammonia liberated in the acid hydrolysis of fraction D should be noted.

For the preliminary chromatographic studies mentioned in the discussion part of this paper, a fraction corresponding

Table 5. *Ionophoretic analysis of a partial hydrolysate of gramicidin*

| Fraction | Comprising | N found (% of total-N in original gramicidin) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Amino-N (4 min. Van Slyke) | | $NH_3$-N | | Hydroxyamino-N | |
| | | Total-N (Kjeldahl) | Tryptophan-N | Before hydrolysis | After hydrolysis | Before hydrolysis | After hydrolysis | Before hydrolysis | After hydrolysis |
| A | Jelly on anode-side of trench | 0·35 | — | — | — | — | — | — | — |
| B | Insoluble material in trench (widely excised) | — | — | — | — | — | 0·7 | — | 0·8 |
| C | Band (3) | — | 1·6 | 4·1 | — | 0·2 | 0·4 | 0·0 | 0·5 |
| D | Band (2) | — | <0·06 | 0·6 | 0·8 | 0·1 | 0·7 | 0·0 | 0·4 |
| E | Intermediate fraction | — | <0·06 | 0·0 | 0·1 | 0·1 | 0·3 | 0·0 | 0·1 |
| F | Band (1) | — | <0·06 | 1·7 | 1·8 | 0·3 | 0·6 | 1·9 | 1·7 |

to Band (2) was obtained, employing in the ionophoresis a jelly made from waterglass with HCl and from which NaCl had been completely displaced by allowing a slow stream of 0·1 N-ammonium acetate to flow over its surface. The ammonium acetate present in the fractions resulting was completely removed by drying them in a high vacuum at room temperature over $H_2SO_4$ and soda-lime. This procedure, evolved by Dr Martin and his colleagues, yields a product substantially free from salt and therefore more suitable for chromatographic study. Migration in the ammonium acetate gel was 2–3 times as rapid as in the sodium acetate gel with the same applied voltage; the bands observed in the prints, however, had much the same positions relative to one another.

## SUMMARY

1. Ethanolamine (2-aminoethanol-1) has been identified as the component of gramicidin hydrolysates which reacts with periodate to yield formaldehyde and $NH_3$.

2. Two ethanolamine residues may occur in the postulated stoichiometric minimum unit of gramicidin. Destruction of ethanolamine during acid hydrolysis of gramicidin is considerable.

3. Attention is drawn to a number of difficulties which arise in relating the over-all properties of intact gramicidin to those which would be expected from study of the products of acid hydrolysis.

4. Preliminary data are given on a basic fraction isolated from a partial acid hydrolysate of gramicidin and thought to contain 'peptides' of ethanolamine.

I wish to thank Drs R. Consden, A. H. Gordon and A. J. P. Martin for much help and advice, especially with the ionophoretic experiments; Mr M. W. Rees for the periodate-aldehyde determinations; Dr M. G. Macfarlane for the P determinations; and Dr D. Crowfoot and Mr G. M. J. Schmidt for crystallographic observations. I am grateful to Messrs H. G. Wellington and W. Wolff for technical assistance.

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# 'Gramicidin S': Over-all Chemical Characteristics and Amino-acid Composition

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(*Received* 12 *June* 1945)

'Gramicidin S' (Soviet gramicidin) is the name given to a crystalline substance of polypeptide character and possessing antibacterial activity, which is elaborated by a strain of *Bacillus* isolated from garden soil. The earlier studies concerning it have been brought together in a publication by Sergiev (1943), of which a short abstract has appeared in English (Gause & Brazhnikova, 1944*a*). More recent data are given by Gause & Brazhnikova (1944*b*), Belozersky & Passhina (1944) and Sergiev (1944).

The present chemical study has extended, and in part confirmed, the preliminary chemical data reported by Belozersky & Passhina. From the data now presented it emerges that gramicidin S is the hydrochloride of a basic peptide, whose stoichiometric minimum unit embodies one residue each of *l*-ornithine, *l*-proline, *l*-valine, *l*-leucine and *d*-phenylalanine, possesses one free basic (amino) group and no free carboxyl groups, and is combined with one residue of chloride. The properties and composition of the substance remain constant on recrystallization.

The relatively fast reaction of the amino-N of gramicidin S with $HNO_2$ reported by Belozersky & Passhina (1944) does not seem an adequate reason for their denial that the amino group is in the side-chain of the ornithine residues, since relatively little is known, so far, of the reaction rates of the amino-N of complicated peptides. Gramicidin S did not evolve $CO_2$ on heating with ninhydrin in acetic acid. The colour reaction with ninhydrin, noted by Belozersky & Passhina and in the present study, does not necessarily signify that free amino groups in the α-position are present, since a wide variety of nitrogenous compounds gives a colour on heating with ninhydrin. In the present study, electrometric titration revealed no groups ionizing in the pH range usually associated with carboxyl groups. Excessive 'protein error' with the indicator employed prevented determination of carboxyl groups by acetone titration. Belozersky & Passhina state that carboxyl groups are present. They appear not to have noticed the presence of chloride in gramicidin S.

Further physical studies will be necessary before it can be decided whether the stoichiometric minimum unit represents the 'true' molecule, or is some submultiple thereof. But whatever the degree of polymerization, and whatever the location of the free amino groups established by future chemical study, it is difficult to escape the conclusion that gramicidin S embodies a 'cyclopeptide' structure with an endless closed peptide chain. Its very high optical rotation may be regarded as supporting this view (cf. Kauzmann, Walter & Eyring, 1940).

The analytical data on which the present conclusions rest are summarized in Tables 2, 3, and 4. They suggest that one molecule of 'bound' water is probably present in dried gramicidin S per stoichiometric unit of 5 amino-acid residues formed from the free amino-acids by elimination of 5 molecules of water. The theoretical requirements in Table 2 are calculated for 0, 1 and 2 such 'bound' water molecules.

The amino-acid data (Tables 3 and 5) present a point of analytical interest. In view of Belozersky & Passhina's report that gramicidin S is hydrolyzed rather slowly with acid, vigorous conditions were employed for hydrolysis, and control experiments with known mixtures of the amino-acids under these conditions showed that some destruction ensued. With tyrocidine hydrolysates excessive variability has been noted in the amino-acid yields (Gordon, Martin & Synge, 1943*c*; Christensen, Uzman & Hegsted, 1945). However, with gramicidin S such variations were not found; correction of the yields on the basis of the recoveries found with known mixtures of amino-acids (Table 5) gave stoichiometric results (Table 3). This suggests that destruction of amino-acids during hydrolysis of gramicidin S is not greatly different from that of the free amino-acids and their acetyl derivatives under the same conditions. This difference between the behaviour of tyrocidine and gramicidin S may tentatively be attributed to the presence of tryptophan in the former.

The chemical data now presented make it clear that gramicidin S is very closely related indeed to tyrocidine. The occurrence, as primary hydrolysis products of these two substances, of both *d*-phenylalanine and *l*-ornithine in itself renders them unique among natural products hitherto described. The relation is made clear by reference to Table 1. It is seen that 2 units of gramicidin S could be embodied as such in 1 unit of tyrocidine. The high negative rotations of both substances may be regarded as indicative of common structure.

Table 1. *Comparison of numbers of residues embodied in stoichiometric minimum units of tyrocidine hydrochloride and gramicidin S*

| | Tyrocidine HCl* | Gramicidin S |
|---|---|---|
| *l*-Ornithine | 2 | 1 |
| *l*-Proline | 2 | 1 |
| *l*-Valine | 2 | 1 |
| *l*-Leucine | 2 | 1 |
| *d*-Phenylalanine | 3 | 1 |
| *l*-Tryptophan | 2 | 0 |
| *l*-Tyrosine | 2 | 0 |
| *l*-Aspartic acid | 2 | 0 |
| *l*-Glutamic acid | 2 | 0 |
| Ammonia | 3 | 0 |
| Chloride | 2 | 1 |

* Hotchkiss (1944); Christensen *et al.* (1945).

Dr R. D. Hotchkiss kindly examined the nature of the antibacterial action of a specimen of gramicidin S and stated (private communication) that it is similar to that of tyrocidine, and different from that of gramicidin (cf. Hotchkiss, 1944). Great chemical similarity to tyrocidine has now been demonstrated. Hotchkiss (1944) attributes the bactericidal action of tyrocidine to the presence of basic and fatty groups in the same molecule, in other words he concludes that it is similar in nature to that of the cationic detergents. Gause & Brazhnikova (1944*b*) report that gramicidin S is more effective than tyrothricin against Gram-negative bacteria. Tyrocidine is the major component of tyrothricin and the one known to be active against Gram-negative organisms. A direct comparison in this laboratory of the activity of gramicidin S and tyrocidine hydrochloride against various strains of *Neisseria* spp. and *Aerobacter aerogenes* in broth containing 10% (v/v) of serum has confirmed that

Table 2. *Determinations on unhydrolyzed gramicidin S*

| | % of dried gramicidin S, Specimen I | | Required for $C_{30}H_{46}O_5N_6 \cdot HCl—X \cdot H_2O$ | | |
|---|---|---|---|---|---|
| | Present work | Belozersky & Passhina (1944) | $X=0$ | $X=1$ | $X=2$ |
| C | 57·5 | — | 59·3 | 57·7 | 56·0 |
| H | 7·6 | — | 7·75 | 7·86 | 7·94 |
| N (Kjeldahl) | 12·9–13·3 | 13·0 | 13·8 | 13·5 | 13·1 |
| Cl (Carius) | 5·6 | — | 5·86 | 5·69 | 5·53 |
| S (Carius) | Nil | — | Nil | Nil | Nil |
| Combustion residue | Nil | — | Nil | Nil | Nil |
| P | 0·07 | — | Nil | Nil | Nil |
| OMe (Zeisel) | Nil | — | Nil | Nil | Nil |
| Chloride (Volhard) | 4·8 | — | 5·86 | 5·69 | 5·53 |
| Amino-N (Van Slyke): 5 min. | — | 1·64 | 2·31 | 2·24 | 2·18 |
| Amino-N (Van Slyke): 30 min. | — | 1·87 | 2·31 | 2·24 | 2·18 |
| Basic groups as N (ethanol titration) (1 equiv. = 1 atom) | — | 1·97 | 2·31 | 2·24 | 2·18 |
| Basic groups (electrometric titration) | 2·0–2·5 | — | 2·31 | 2·24 | 2·18 |
| Carboxyl groups (electrometric titration) | Nil | Present | Nil | Nil | Nil |
| α-Amino groups (ninhydrin-$CO_2$) | Nil | — | Nil | Nil | Nil |
| Tryptophan (colorimetric) | Nil | Nil | Nil | Nil | Nil |

Table 3. *Determinations on acid hydrolysates of gramicidin S*

| | As % of total N of gramicidin S | | | |
|---|---|---|---|---|
| | Present work | | | |
| Determination | Gramicidin S Specimen I | Gramicidin S Specimen II | Belozersky & Passhina (1944) | Theoretical |
| Amino-N (30 min. Van Slyke) | 84·7 | — | 90·1 | 83·3 |
| α-Amino-N (ninhydrin-$CO_2$) | 79·0 | — | — | 83·3 |
| $NH_3$ | 0·2 | — | 0·7 | Nil |
| Hydroxyamino-N | 0·2 | — | — | Nil |
| Humin-N | — | — | 0·3 | — |
| Basic amino-acid N | — | — | 27·0 | 33·3 |
| Ornithine-N | 33·0 | 33·6 | 29 | 33·3 |
| Proline-N | 16·7 | 16·8 | 9–14 | 16·7 |
| Valine-N | 17·1 | 17·1 | — | 16·7 |
| Leucine-N | 16·8 | 17·3 | About 33* | 16·7 |
| Phenylalanine-N | 16·6 | 16·1 | Nil | 16·7 |
| Tyrosine (colour reaction) | Nil | — | Nil | Nil |
| Other usual protein constituents (Consden, Gordon & Martin, 1944) | Nil | — | Various tests negative | Nil |

* The leucine figure of 70% of Belozersky & Passhina (1944) is a misprint for 40%.

gramicidin S is much the more effective of the two. This may tentatively be attributed to its higher relative content of basic (ornithine) residues.

The name gramicidin S conveys correctly the fact that the new peptide has a similar origin and mode of preparation to the gramicidin of Dubos and Hotchkiss (see Hotchkiss, 1944). However, it is confusing for a number of reasons: chemically the substance contains only one species of residue (*l*-valine) in common with gramicidin; it possesses, unlike gramicidin, considerable antibacterial activity against Gram-negative organisms; it appears to exert its antibacterial activity by virtue of a different mechanism. In all these respects the new peptide has close affinities with tyrocidine. It is to be hoped that the discoverers will consider renaming it in such a way as to bring out these affinities.

Gramicidin S provides an interesting addition to our knowledge of the polypeptides synthesized by *Bacillus* spp. Structurally, it may turn out to be simpler than gramicidin and tyrocidine, and study of it, apart from its intrinsic interest, may prove helpful for structural studies of tyrocidine.

## EXPERIMENTAL

### *Material used and constancy of properties on recrystallization*

The first specimen of gramicidin S studied was crude or 'clinical' material in aqueous-ethanolic solution, received through the Medical Research Council; from this, crystalline material was readily prepared by crystallization from aqueous ethanol after removal of fat (cf. Sergiev, 1943). The once recrystallized product cannot have been pure, since two-dimensional partition chromatograms on paper with an acid hydrolysate (Consden, Gordon & Martin, 1944) revealed the presence of faint spots corresponding to amino-acids other than those eventually found in the pure material.

The specimen employed for most of the studies reported here was crystalline material, as received from Prof. P. G. Sergiev, Institute of Tropical Medicine, Moscow. This material is here designated I. Specimen II was obtained from I by dissolving it in 70% (v/v) aqueous ethanol, and allowing crystallization to occur as the solution evaporated at room temperature. A loss of 60% was permitted in the recrystallization. Specimen II had the same N content, optical rotation (see below) and amino-acid composition (Table 3) as specimen I. Gramicidin S is notably more soluble in aqueous than in absolute ethanol.

*Optical rotation.* This was determined in 70% (v/v) aqueous ethanol ($l=0{\cdot}5$, $c=1{\cdot}5$):

Specimen I: $[\alpha]_D^{18°}$, $-292°$. Specimen II: $[\alpha]_D^{20°}$, $-295°$.

### *Chemical analyses on intact gramicidin S*

The results are shown in Table 2. Air-dry gramicidin S loses 3–5% by weight of moisture on drying over $P_2O_5$ *in vacuo* at 100°. Analyses on intact gramicidin S are referred to material dried in this way. C, H, S, Cl (Carius) and OMe determinations were by Dr G. Weiler, Oxford. P was determined by Dr M. G. Macfarlane colorimetrically after incineration with $H_2SO_4$ and perhydrol. N (Kjeldahl) determinations were done with 6 hr. incineration in the mixture of Campbell & Hanna (1937).

*Chloride (Volhard) titrations.* 5–10 mg. gramicidin S (dissolved in 1·5 ml. 70% ethanol) were treated with 2 ml. 0·02N-$AgNO_3$ (made up in approx. 2N-$HNO_3$) at room temperature, and back-titrated with 0·02N-KCNS in the presence of iron alum. The rather low results may be attributed to partial precipitation of gramicidin S with Ag under these conditions.

*Tryptophan.* This was determined colorimetrically with *p*-dimethylaminobenzaldehyde in acetic acid-HCl mixtures (cf. Synge, 1944).

*Ninhydrin-$CO_2$ determination.* This was done according to Van Slyke, Dillon, MacFadyen & Hamilton (1941) in 1 ml. glacial acetic acid with 50 mg. ninhydrin, and heating at 100° for 5 min. Gramicidin S (16 mg.), on being heated under these conditions, gave a bright red colour. The $CO_2$ resulting was not significantly different from the reagent blank.

*Electrometric titration.* This was done with a Cambridge pH meter, fitted with glass electrode. 100 mg. gramicidin S were suspended in 10 ml. water. On titrating with 0·1N-HCl, the pH curve was not significantly different from that for the water blank. The solution had initial pH 3·7. The addition of NaOH equivalent to the HCl added brought the pH back to this value. Some crystallization from the solution of gramicidin S was noted during the addition of HCl. Presumably this was a solubility product effect. When more 0·1N-NaOH was added, the pH rose above 6 with the first drop; maximum buffering seemed to occur at about pH 8·5. The material in suspension swelled and became gelatinous and lumpy, in such a way that the centres of the lumps could not react with the alkali. Repeated neutralization of the alkali with 1 equiv. HCl (which brought the pH back to its original value) was followed by rendering alkaline again with stirring. This broke up the lumps and appeared to bring all the material into a reactive condition; by comparison with the water blank an estimate could be formed of the free basic groups of gramicidin S (see Table 2).

### *Acid hydrolysis of gramicidin S*

Hydrolysis was effected in sealed evacuated tubes with excess of a 1 : 1 (v/v) mixture of glacial acetic acid and 10N-HCl for 48 hr. at 110–115°. The chromatographic studies of hydrolysates prepared in this way gave no indications of peptides that had escaped hydrolysis. The hydrolysates contained a little humin, which was absent from mixtures of the known amino-acid constituents of gramicidin S treated in the same way. Before further study, hydrolysates were evaporated to dryness *in vacuo*, made up to known volume with water, and stored at 0°.

### *Study of acid hydrolysates of gramicidin S*

The results are shown in Tables 3 and 4.

*Amino*-N was determined by the Van Slyke manometric procedure with 30 min. reaction time. No yellow colour was noted in the reaction mixture, the absence of tyrosine from the hydrolysate thus being confirmed.

*Ninhydrin-$CO_2$.* The method of Van Slyke *et al.* (1941) was employed. With both pH 2·5 and 4·7 citrate buffers the same result was obtained, and the low value may have its origin in the destruction of amino-acids during the

hydrolysis, noted in the determinations of individual amino-acids.

$NH_2$- *and hydroxyamino*-N. These determinations were carried out by the Conway modification of Van Slyke's procedure described in the preceding paper (Synge, 1945).

*Identification of individual amino-acids in hydrolysate.* Drs Consden, Gordon & Martin examined the hydrolysate of gramicidin S (specimen I) for amino-acids by their (1944) two-dimensional partition chromatographic method with paper, employing phenol and collidine for development. Only five components were noticed, corresponding in rates to the five amino-acids subsequently shown to be present. Special control experiments with butanol-benzyl alcohol mixtures on one-dimensional paper chromatograms showed that the leucine component was free from *iso*leucine. The results with paper chromatograms were confirmed by preliminary partition-chromatographic fractionation of acetylated hydrolysates on silica gel, done in this laboratory and employing *cyclo*hexane-propanol-$H_2O$ and chloroform-butanol-$H_2O$ systems successively.

The isolation of the five amino-acid components was then undertaken. An acid hydrolysate of 100 mg. gramicidin S (I) was first fractionated by the ionophoretic procedure of Consden, Gordon & Martin (which is mentioned in the preceding paper (Synge, 1945)). Sodium acetate jelly was employed at pH 6. Two well-defined fractions resulted, the ornithine migrating towards the cathode faster than the other amino-acids. The two fractions were worked up and subjected separately to the acetylation and extraction procedure described by Gordon, Martin & Synge (1943*a*). In the case of the ornithine fraction, the evaporated extract yielded crystals of diacetylornithine without further treatment. The product from the other ionophoretic fraction was submitted to partition chromatographic fractionation on silica gel chromatograms. With 5% propanol-*cyclo*hexane the 'leucine-phenylalanine' fraction was allowed to run out of the chromatogram. The solvent was then changed to 30% propanol-*cyclo*hexane and the 'valine' and 'proline' fractions were run out and collected separately. After evaporation, the 'valine' and 'proline' residues were freed from indicator (peonin) by filtration, in 17% butanol-chloroform, through a little water-saturated silica gel (cf. Gordon *et al.* 1943*c*). The 'leucine-phenylalanine' residue was resolved by chromatographic fractionation with butanol-chloroform on silica gel employing the '*R*—$NH_4$' indicator of Liddell & Rydon (1944). All the resulting fractions were evaporated to dryness, giving the corresponding acetylamino-acids in approximately the expected yields.

The optical rotation of each of the five fractions containing the acetylamino-acids was then determined. It seemed preferable, departing from previous practice (cf. Gordon, Martin & Synge, 1943 *b*, *c*; Synge, 1944), to determine this before crystallizing, since the process of crystallization might lead to fractionation of optical isomers. The amount of acetylamino-acid, on which the calculation of specific rotation was based, was determined by titrating a sample of the solution used for polarimetry with 0·01 N-$Ba(OH)_2$ to bromthymol blue in the usual way. The residues from the optical determinations were then used for crystallizing and characterizing the acetylamino-acids with the results shown in Table 4. Data on the authentic reference compounds and on corresponding fractions obtained from tyrocidine are given by Gordon *et al.* (1943*c*). Reference to

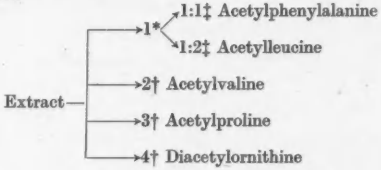
Table 4. *Characteristics of acetylamino-acids isolated from hydrolysate of gramicidin S*

| Compound | Formula | Optical rotation: $[\alpha]_D$ | °C | $l$=0·5 $c$= | Solvent | Crystallized from | Uncorrected: m.p. | Mixed m.p. | m.p. of authentic material used in mixed m.p. determination | % C: Found | % C: Calc. | % H: Found | % H: Calc. | % N: Found | % N: Calc. | Acid equiv. wt.: Found | Acid equiv. wt.: Calc. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *NN'*-Diacetyl-*l*-ornithine | $C_9H_{16}O_4N_2$ | + 3° | 20·5 | 1·8 | Ethanol | Ethyl acetate | 156° | 157° | 157° | 50·6 | 50·0 | 7·6 | 7·4 | 13·0 | 13·0 | 216 | 216 |
| Acetyl-*l*-proline | $C_7H_{11}O_3N$ | −118° | 22 | 1·0 | Water | Wet ethyl acetate | 83°* | 82°* | 81°* | 54·1 | 53·5 | 7·2 | 7·0 | 7·7* | 8·0* | 174* | 175* |
| Acetyl-*l*-valine | $C_7H_{13}O_3N$ | + 4° | 21·5 | 1·2 | Ethanol | Chloroform-light petroleum | 163° | 165° | 168° | 54·2 | 52·8 | 7·9 | 8·2 | 8·5 | 8·8 | 156 | 159 |
| Acetyl-*l*-leucine | $C_8H_{15}O_3N$ | − 28° | 20 | 1·2 | ,, | ,, | 182° | 185° | 186° | 54·8 | 55·5 | 8·6 | 8·7 | 7·7 | 8·1 | 175 | 173 |
| Acetyl-*d*-phenylalanine | $C_{11}H_{13}O_3N$ | − 48° | 20 | 1·3 | ,, | ,, | 167° | 165° | 164°† | 64·8 | 63·7 | 6·8 | 6·3 | 6·6 | 6·8 | 206 | 207 |

* Determined with air-dry hydrate, $C_7H_{11}O_3N.H_2O$. † Material isolated from tyrocidine (Gordon *et al.* 1943*c*).

this paper will show that the acetylamino-acids other than diacetylornithine isolated in the present study possessed fairly high optical rotations indicating that little racemization could have occurred during hydrolysis and isolation. The configuration of two of the acetylamino-acids from gramicidin S (acetyl-*d*-phenylalanine and acetyl-*l*-leucine) was further checked, since admixture with authentic specimens of their optical antipodes caused considerable (about 20°) depression of m.p.

*Quantitative determination of amino-acids in hydrolysate.* Quantitative analyses were conducted on hydrolysates of approximately 10 mg. gramicidin S by the general procedure of Gordon *et al.* (1943*a*). The 'separating funnel' acetylation and extraction procedure was employed. This gave low but reproducible recoveries of ornithine. The scheme of fractionation shown in Fig. 1 was employed with the acetylated extract.




* Fraction run out in 5% propanol-*cyclo*hexane.
† Fraction run out in 30% propanol-*cyclo*hexane.
‡ Fraction run out in 3% butanol-chloroform.

Fig. 1.

For the chromatograms, silica gel batch $\gamma$ was used throughout; with the *cyclo*hexane chromatograms, peonin chloride was used as indicator, and with the chloroform chromatograms, methyl orange (Gordon, Martin & Synge, 1944).

The analyses were controlled by subjecting a mixture, in the correct quantities, of acetylamino-acids to the same hydrolytic and analytical procedures. Percentage recoveries in two separate experiments are shown in Table 5.

The values shown in Table 5 have been used as the basis for correcting the yields in the analyses of gramicidin S hydrolysates; the corrected figures for analyses of specimens I and II appear in Table 3.

Table 5. *Recovery of amino-acids in control experiments*

| Material hydrolyzed and analyzed | Percentage recovery (*a*) | (*b*) |
|---|---|---|
| Acetyl-*dl*-phenylalanine | 93 | 92 |
| Acetyl-*dl*-leucine | 95 | 94 |
| Acetyl-*dl*-valine | 95 | 95 |
| Acetyl-*l*-proline | 103 | 101 |
| *NN'*-Diacetyl-*l*-ornithine | 70 | 72 |

Direct chromatography of the mixture of acetylamino-acids used in the control experiments led to recoveries within 3% of theory; it is therefore to be assumed that destruction occurs during the hydrolytic treatment, since the acetylation and extraction procedure gives quantitative recovery of the amino-acids other than ornithine (Gordon *et al.* 1943*a*, *b*; Synge, 1944).

## SUMMARY

1. It is probable that the antibacterial substance gramicidin S is a cyclopeptide hydrochloride having a stoichiometric minimum unit formed from one residue each of *l*-ornithine, *l*-proline, *l*-valine, *l*-leucine and *d*-phenylalanine. The unit possesses one free amino-group, no free carboxyl groups and one residue of chloride.

2. The relationship of gramicidin S to tyrocidine hydrochloride is emphasized, and a plea is made for renaming gramicidin S to bring out this relation.

I wish to thank Prof. P. G. Sergiev, Moscow, and the Soviet Red Cross for a supply of gramicidin S and for information about it; Drs R. Consden, A. H. Gordon, and A. J. P. Martin, Leeds, for carrying out the chromatographic experiments with paper; and Dr M. G. Macfarlane for P determinations. I am grateful to Mr W. Wolff for his technical assistance.

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# An 'Ammonia'-yeast and some of its Properties

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(*Received* 12 *June* 1945)

An account is given here of the manner of preparing a living yeast in which all the potassium is replaced by ammonia, the process being reversible. The fermenting power, growth and resting oxygen uptake of such yeast are described.

## METHODS

### *Chemical*

*Ammonia* was determined by a microdiffusion method (Conway, 1939).

*Potassium* was determined as previously described (Boyle, Conway, Kane & O'Reilly, 1941).

### *Preparation of the 'ammonia'-yeast*

Ordinary bakers' yeast (as supplied from the Cork Yeast Factory) was used at the outset; then a pure strain (yeast 'race 12') was investigated and found to behave in a similar manner, but the bakers' yeast was preferred for the investigation as it concentrated K and ammonia better. A pure strain was then grown from the commercial bakers' yeast by plating in agar containing malt extract, and selecting one colony for further growth in Pasteur flasks. This yeast was used in most of the experiments. The growth medium was made up as follows: glucose, 12·5 g.; sucrose, 12·5 g.; $KH_2PO_4$, 10 g.; $MgSO_4$, 1 g.; yeast extract, 250 ml. The pH was adjusted with NaOH to 5·65 and tap water added to give 1000 ml. The yeast extract was made by boiling 100 g. yeast with 500 ml. water for 1 hr., cooling, filtering and making up to 500 ml.

The process of ammoniating the yeast consists in suspending 1 part of yeast in 50 parts of a medium having the following composition: 363·0 ml. of 1·17% NaCl; 10·0 ml. of 2% $CaCl_2$; 20 ml. of 5% $NaHCO_3$; 10 ml. of 7% glucose; 5 ml. of an equal mixture of 0·02 M-$Na_2HPO_4$ and 0·02 M-$NaH_2PO_4$. This was made up to 1 l. with 0·2 N-$NH_4Cl$. Another suspension of the same yeast was made in a solution similar to the above, but in which KCl replaced $NH_4Cl$. The yeast was maintained in suspension by bubbling with a gas mixture containing 3% $CO_2$ and 97% $O_2$. The solution was at first renewed at intervals of some hours and then left overnight for a 24 hr. period. In later experiments bubbling was suspended in the evening and continued next day. With continued bubbling almost all the K may be removed after 1 day's treatment, but may require 2 days or longer for the traces. Longer periods are also required for the interrupted bubbling.

### *Examples of the* K *removal and reversal*

The sample of bakers' yeast was first washed in Ringer fluid and centrifuged. Analysis of the centrifuged yeast showed 450 mg./100 g. of K and 1 mg./100 g. of ammonia-N. The yeast was then suspended in the fluid described above for the replacement of K by $NH_3$; two other suspending fluids containing 0·05 N- and 0·01 N-$NH_4Cl$ were also used. The yeast was kept suspended by bubbling with the gas mixture containing 3% $CO_2$. After 24 hr. the analyses of the centrifuged ammonia-yeast were as follows:

| | Normality of $NH_4Cl$ in suspending fluid | | |
|---|---|---|---|
| | 0·2 | 0·05 | 0·01 |
| K content of yeast (mg./100 g.) | 0 | 192 | 268 |
| Ammonia-N of yeast (mg./100 g.) | 400 | 174 | 51 |

Each preparation of yeast was then washed in a similar fluid containing no $NH_4Cl$ but 0·033 N-KCl, and the gas mixture sent through the fluid as before for 24 hr. The analyses of these K-yeasts showed:

| | Normality of $NH_4Cl$ in previous suspending fluid | | |
|---|---|---|---|
| | 0·2 | 0·05 | 0·01 |
| K content of yeast after suspension in KCl containing fluid (mg./100 g.) | 425 | 455 | 436 |
| Ammonia-N content (mg./100 g.) | 47 | 17 | 7 |

The following analyses are for two samples of K-yeast and two of ammonia-yeast, the yeast being a pure strain grown from a selected colony after plating bakers' yeast. These yeasts had been suspended for 4 days, with bubbling interrupted overnight. One solution contained 0·2 N-KCl and the other 0·2 N-$NH_4Cl$.

| | K-yeast | | Ammonia-yeast | |
|---|---|---|---|---|
| K content (mg./100 g.) | 662, | 775 | 2·1, | 0·0 |
| Ammonia-N (mg./100 g.) | 0·0, | 1·1 | 320, | 348 |
| P (mg./100 g.) | 265, | 313 | 265, | 313 |

A striking feature of this complete replacement of K by ammonia is that it does not occur in the absence of $CO_2$, when the suspending fluid is maintained at the same pH. To demonstrate this the bicarbonate in the suspending fluid was replaced by an equivalent amount of NaCl, the gas mixture used for bubbling being pure oxygen. Fig. 1 shows the contrast of entrance of ammonia with and without the bicarbonate-$CO_2$ system. The upper curve is that of medians from five experiments and the lower curve

is that of means from two, each of which gave almost identical results. The pH for both curves was 7·3.

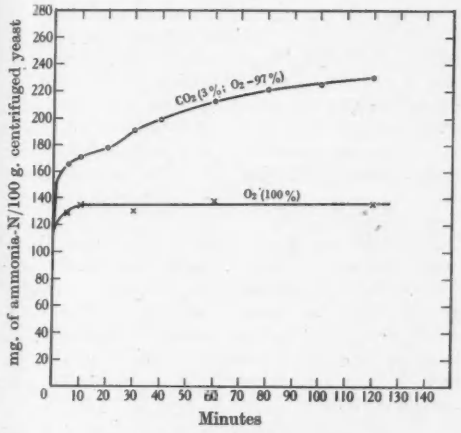


Fig. 1. Ammonia content of centrifuged yeast. The upper curve is for a yeast suspension through which $CO_2$ : $O_2$ mixture had been passed, in the presence of bicarbonate (pH 7·3). The lower curve is for a similar suspension treated with $O_2$ without $CO_2$ or bicarbonate, but at the same pH.

The centrifuged yeast used for the analyses contained a relatively large volume of interspace fluid. As measured by inulin this amounted to 20–30%, but it will be seen that with the oxygen bubbling the ammonia 'space' is greater than this, though ammonia does not continue to enter. The curve with $CO_2$ appears to show a marked inflexion at about 20 min.

After 24 hr. of bubbling with 3% $CO_2$ in $O_2$ the yeast suspended in bicarbonate-containing medium had approximately 330 mg. ammonia-N/100 g., while that suspended in bicarbonate-free medium and bubbled with $O_2$ contained 153 mg. ammonia-N/100 g.—this latter being only slightly higher than the figure for 2 hr.

### *Some properties of the ammonia-yeast*

*Fermentation.* In the fermentation studies 0·5 ml. of 6% glucose was introduced into each of a series of Conway units (no. 1) and then 1 ml. of 0·5N-NaOH containing 5% Universal Indicator (British Drug Houses Ltd.) into the central chambers. As each delivery of the alkali was made a lid smeared with vaseline or liquid paraffin was placed in position. Then 0·5 ml. of the yeast suspension was introduced quickly into the outer chamber, with the lid displaced a little for the purpose, the lid being immediately replaced in position. The contents were mixed by rotation. The yeast suspension consisted of 1 part of yeast in 10 of water. (In some experiments the suspending fluid was 10 ml. of 0·43% ammonium acid phosphate, or alternatively 0·50% $KH_2PO_4$ for the K-yeast, and 0·1% $MgSO_4$, but these gave essentially the same results.) At varying times after the introduction of the yeast suspension the lids were detached, 0·5 ml. of saturated $BaCl_2$ added to the outer chamber and the titration (to a light green) carried out with N-$H_2SO_4$ from the Conway burette. In such fermentation the $CO_2$ absorption very quickly becomes in linear relation with time, and then indicates the rate of fermentation.

A similar series was set up with a 1 in 10 suspension of yeast, which was either K-yeast treated similarly throughout, except that K replaced ammonia, or the ammonia-yeast suspended in a solution containing some added $KH_2PO_4$, the K concentration being either 23 mg./100 ml. or 93 mg./100 ml. The comparison made in such a way showed throughout a similar relationship between fermentation by the ammonia-yeast and that by the K-yeast or the ammonia-yeast with added potassium. In the absence of K, fermentation proceeded at about 40% of the rate with K present. The relation is shown in Fig. 2. The curves in Fig. 2 are of interest

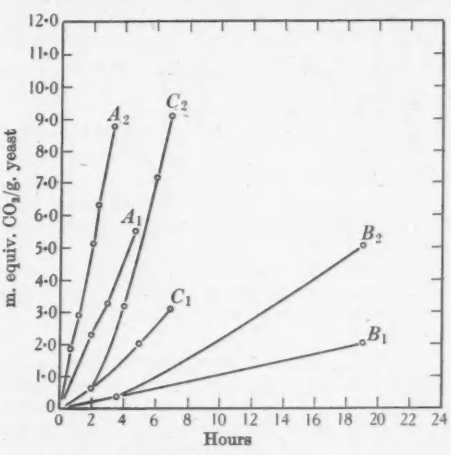


Fig. 2. Fermentation rates of yeast in 1 in 20 suspension, containing 3% glucose. $A_1$ and $A_2$, samples of ammonia-yeast and K-yeast. $B_1$ and $B_2$, samples of ammonia-yeast and ammonia-yeast plus K (11·5 mg./100 ml. in the final mixture). $C_1$ and $C_2$, for the same ammonia-yeast suspension as $B_1$ and $B_2$ but containing 10% ammonia-yeast extract.

in that fermentation with samples $A_1$ and $A_2$ (an ammonia and a similarly treated K-yeast) is comparatively rapid, and with samples $B_1$ and $B_2$ (an

ammonia-yeast and the same with K added to the suspension) relatively slow. The ratio of fermentation is, none the less, very similar. The samples $C_1$ and $C_2$ are for the same yeast suspensions as $B_1$ and $B_2$ but the fermentation rate has been considerably increased by incorporating in 100 vol. of the suspension 20 vol. of yeast extract. Such yeast extract was prepared as under Methods, but from an ammonia-yeast when the fermentation of such was being studied, and from a K-yeast when this was being examined.

In samples B and C there is a noticeable initial lag, but K addition has not removed this lag, while considerably increasing the subsequent rate.

*Growth experiments.* In the growth experiments the ammonia-yeast was compared with a K-yeast treated similarly throughout with K instead of ammonium ions, or with the same ammonia-yeast in suspension. The result compared with the K-yeast will be first described.

*Growth media.* For the K-yeast the medium contained: 2·5 g. glucose; 0·50 g. of $KH_2PO_4$; 0·10 g. of $MgSO_4$ (anhyd.) and 20 ml. of yeast extract (made as described under Methods) to 100 ml. The pH was 5·2, and the mixture was sterilized in the autoclave.

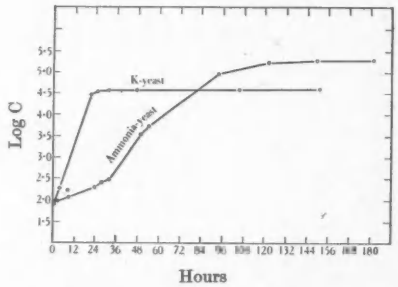


Fig. 3. Growth curves of ammonia-yeast and companion K-yeast. C is the number of yeast cells/cu.mm.

For the ammonia-yeast a similar medium was used, the K salt being substituted by equivalent amounts of the $NH_4$ salt, and the yeast extract was made from ammonia-yeast prepared as already described.

For seeding the yeasts for growth, 0·1 g. of the K-yeast, for example, was suspended in 10 ml. suspending fluid (containing 0·50 % $KH_2PO_4$ and 0·1 % $MgSO_4$ as mentioned above under fermentation studies), and 0·1 ml. of this added to the above media in a Pasteur flask, a similar procedure being used for the ammonia-yeast, ammonium ions replacing K ions throughout. A count of yeast cells/cu.mm. was made with the haematocytometer counting slide, and then the flask maintained at 28°, such counts being made at intervals up to about a week.

The results are shown in Fig. 3 in which the logs of the counts/cu.mm. are plotted against the time.

In the first 24 hr. the growth of the ammonia-yeast is only a small fraction of that of the K-yeast. During the second 24 hr. there is a rapid increase and on the third day the number of cells begins to exceed that of the K-yeast. This latter ceases growth after 2 days, the cell count being then about 46,000/cu.mm., whereas the ammonia-yeast continues growing up to the seventh day and ends with cells four times as numerous, but much smaller in size than the K cells.

*Effect on growth of small* K *additions to the ammonia-yeast suspension.* Comparisons were made with parts of the same ammonia-yeast in similar growth media (as described above), to which small amounts of $KH_2PO_4$ were added. When such additions were made, equivalent additions of the ammonium salt were made to the suspension containing no K.

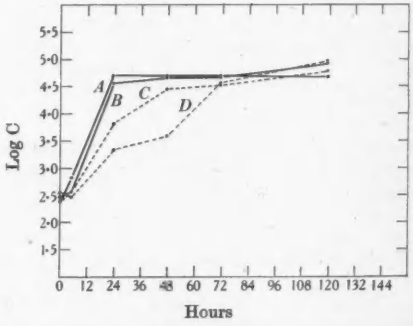


Fig. 4. Growth curves of ammonia-yeast. A and B suspensions contain 11·5 and 46·5 mg. K/100 ml. D and C are for the same ammonia-yeast suspensions without K added, but containing equivalent additions of $(NH_4)H_2PO_4$.

The results are shown in Fig. 4. The curves A and B are for 94 and 23 mg. K/100 ml. suspension, and it will be seen that both are nearly identical and agree closely with the curve for the K-yeast in Fig. 3. Curves D and C are for suspensions in which additions of the ammonium salt equivalent to the K additions of 23 and 94 mg. K/100 ml. were made to the growth media.

It will be seen that comparatively small amounts of K bring the ammonia-yeast to a growth rate and final level similar to the K-yeast, and further that the ammonia preparation behaves again somewhat as in Fig. 3, but that the increased additions of ammonium hydrogen phosphate tend to advance the growth rate to near that of the K preparation.

*Oxygen uptake of the resting ammonia- and K-yeasts.* 100 mg. of ammonia- or of K-yeast were suspended in 10 ml. of a medium containing 0·095 % $Na_2HPO_4$, 0·080 % $NaH_2PO_4$ and 0·60 % NaCl. The pH was 6·7. 3 ml. of this suspension were placed in the Warburg apparatus, the direct method for the estimation of $O_2$ consumption being used. Any $CO_2$ produced was absorbed in KOH solution in the central chamber. The temperature was maintained at 30° and the rate of shaking at 120 oscillations/min.

Table 1. *Oxygen uptake by resting ammonia- and K-yeasts*

| Ammonia-yeast | | K-yeast | |
|---|---|---|---|
| Time (min.) | $O_2$ uptake (cu.mm.) | Time (min.) | $O_2$ uptake (cu.mm.) |
| 0 | 0·0 | 0 | 0·0 |
| 10 | 10·7 | 10 | 5·0 |
| 20 | 21·5 | 20 | 15·1 |
| 30 | 32·2 | 30 | 20·1 |
| 40 | 41·1 | 40 | 25·1 |
| 50 | 55·5 | 50 | 31·9 |
| 60 | 62·6 | 60 | 33·5 |
| 70 | 76·9 | 70 | 38·6 |

The amount of dry matter in the samples of ammonia- and of K-yeast was in each case 7·05 mg.

Table 1 illustrates the results obtained. The dry matter in the sample of yeast used was, in each case, 7·05 mg., so that from the curve of $O_2$ uptake the $Q_{O_2}$ for the K-yeast $= -5·1$, and $Q_{O_2}$ for the ammonia-yeast $= -9·2$. The ammonia-yeast shows a considerably greater oxygen uptake than the K-yeast.

## DISCUSSION

In the above experiments it is shown that the $K^+$ of yeast can be entirely replaced by $NH_4^+$ and the ammonia-yeast so formed can be again converted to a K yeast. Essential to the interchange of ammonia and K is the presence of $CO_2$ (which used at a pH of 7·3 necessitates in turn very appreciable bicarbonate concentration). This would seem to indicate that the ammonia may cross the membrane in the form of carbamic acid, ammonium carbamate, or related substance, which dissociates inside the yeast cell.

The experiments show that $K^+$ is not necessary for fermentation, but that compared with $NH_4^+$ ions, those of potassium have a pronounced stimulating action on the process. Growth can also occur in the absence of K, but there are striking differences between the rate of growth of the ammonia-yeast as compared with that of the K-yeast. Similar differences exist between the growth of the ammonia-yeast in media containing no K, and one to which some K is added. Little further effect is produced when the suspending fluid contains 23 mg. of K/100 ml.

The increased number of cells produced in a given medium by the ammonia-yeast, and the distortion of shape are likewise of interest.

The fact that the 'resting' ammonia-yeast shows a greater $O_2$ uptake is perhaps associated with the fact that ammonium salts can be utilized by the cells as their source of nitrogen.

## SUMMARY

1. The K of living bakers' yeast can be entirely replaced, under certain conditions, by ammonia. This process can be then reversed, the K being restored.

2. The ammonia-yeast ferments glucose to about 40 % the rate of the K-yeast.

3. It can grow in the absence of K, but the growth rate is much slower in the earlier stages. After some days without change of medium the number of cells produced by the K-yeast reaches a limit which is later much exceeded by the ammonia-yeast. The latter produces smaller cells, which have a tendency to form branching chains.

4. The oxygen consumption of the ammonia-yeast is higher than that of the K-yeast.

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# Seasonal Variation of Carotene and Vitamin A in Butter-fat and in Serum

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(*Received* 13 *June* 1945)

During the past few years, numerous papers have appeared dealing with the seasonal variation of either the vitamin A activity of butter or the carotene and vitamin A content of blood serum. For instance, Koehn (1939, 1941) showed that the activity of butter-fat followed a definite cycle, decreasing steadily from August to December, and then increasing threefold between January and June. Sen & Sarkar (1942), feeding carotene at the level of 870,000 i.u./day, obtained a butter-fat of potency 21·6 i.u./g. in January. When the animals were transferred to a diet low in carotene, the carotene of the butter-fat dropped at once and the vitamin A *per se* decreased more slowly. An interesting observation made by these workers was that the contribution of the carotene, which was normally about 27% of the total vitamin A activity of the fat, could not be increased above 35% even by feeding green food. Berl & Peterson (1943) found potencies of 22 and 40 i.u./g. of fat in January and July–September respectively.

Most of the data on serum vitamin A are concerned with young or new-born calves, in which the serum vitamin A is very low (Moore, 1942). Early work by Gillam & El Ridi (1935) showed a marked seasonal variation in serum carotene and vitamin A of cows, winter figures being 0·4 mg. and 400 i.u./100 ml. for carotene and vitamin A respectively, and the corresponding summer figures being 1·10 mg. and 900 i.u./100 ml. A recent paper by Braun (1945) gives for pasture-fed cows carotene values which decrease steadily from 1·35 mg./100 ml. in March to 0·3 mg. or less in September, followed by an increase to 1·0 mg. between September and January.

It is becoming apparent that of the ways of assessing vitamin A deficiency, the measurement of serum carotene and vitamin A is the most sensitive. Lewis, Bodansky, Falk & McGuire (1942), using rats as experimental animals, have shown that an intake of only 2 i.u./day is sufficient to maintain normal dark adaptation, and 25 i.u./day will support normal growth, but to maintain serum level of 100–112 i.u./100 ml., which is considered compatible with high liver storage and satisfactory vitamin A nutrition, 50 i.u./day must be supplied.

The results obtained in the present work are reported partly because there does not appear to be any paper giving both butter-fat and serum results for the same cows at about the same time, partly because of the increasing importance of serum measurements, and partly because the serum values obtained, while agreeing qualitatively with those of Braun (1945), show a different seasonal distribution.

## EXPERIMENTAL

### *Animals and feeding practice*

The herd consisted of 70 pure-bred Ayrshire cattle at the farm of J. Bibby and Sons, Ltd., at Puddington, Cheshire. The animals were 4–5 years old and at various stages of lactation. From April until the end of October, they were grazing on permanent pasture of average quality. From November until the middle of December, the animals were out at pasture during the day only, and afterwards they were in stalls continuously until the following April. Stall-feeding consisted of kale, hay and roots with concentrates to maintain milk yield.

The butter samples, prepared from milk from the entire herd, were kindly supplied by Miss R. A. Spence. The blood samples were drawn by the staff of the University of Liverpool Veterinary Field Station, Willaston, by the courtesy of Prof. Wright. They were taken at about monthly intervals from ten cows at random; 25 or 50 ml. of the pooled serum were treated as described below.

### *Methods*

Butter samples of known moisture content were treated using method B described by Morton, Lord & Goodwin (1941). Results were calculated in i.u./g. of fat for both carotene and vitamin A, and the total vitamin A activity was computed as the sum of these amounts.

The samples of serum were treated by the method of Yudkin (1941), followed by saponification and separation of the carotene by passing over a bone-meal column as described by Mann (1943). After removal of the carotene, the vitamin A was eluted using a mixture of equal volumes of light petroleum (40–60°) and acetone, and finally acetone alone. The results are calculated in mg./100 ml. for the carotene, and in i.u./100 ml. for the vitamin A.

## RESULTS AND DISCUSSION

### *Butter-fat*

The results (Table 1 and Fig. 1) represent vitamin A activity of butter samples examined between August 1941 and August 1943. During the first period of

Table 1. *Seasonal variation of vitamin A activity of butter-fat*

| | I.u./g. of fat | | | | | |
|---|---|---|---|---|---|---|
| | 1941–2 | | | 1942–3 | | |
| | Carotene | Vitamin A | Total | Carotene | Vitamin A | Total |
| August | 7·7 | 27·2 | 34·9 | 8·6 | 23·7 | 32·3 |
| September | 7·5 | 22·4 | 29·9 | 7·8 | 22·4 | 30·2 |
| October | 6·7 | 24·2 | 30·9 | 8·2 | 23·3 | 31·5 |
| November | — | — | — | 9·2 | 24·1 | 33·3 |
| December | 6·5 | 24·3 | 30·8 | 6·2 | 15·6 | 21·8 |
| | Stall-feeding started | | | | | |
| January | 5·5 | 16·4 | 21·9 | 3·7 | 11·4 | 15·1 |
| February | 3·8 | 13·4 | 17·2 | 2·8 | 8·7 | 11·5 |
| March | 2·5 | 11·8 | 14·3 | 2·4 | 12·0 | 14·4 |
| April | 2·7 | 11·7 | 14·4 | 2·7 | 10·7 | 13·4 |
| | Pasture-feeding started | | | | | |
| May | 7·3 | 18·4 | 25·7 | 12·5 | 24·6 | 37·1 |
| June | 10·0 | 20·4 | 30·4 | 12·4 | 17·9 | 30·3 |
| July | 9·1 | 21·2 | 30·3 | 9·2 | 19·2 | 28·4 |

12 months, samples were examined at weekly intervals, and the average for each month is presented in the table; during the second period, samples were examined at intervals not exceeding 1 month.

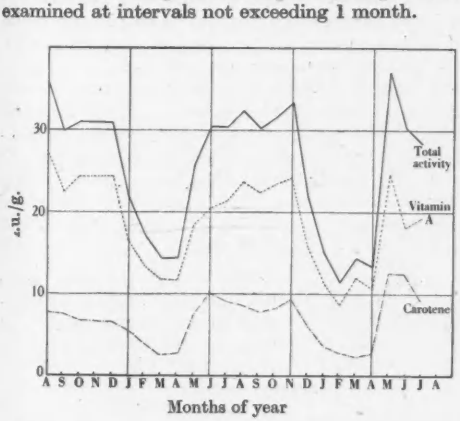


Fig. 1. The seasonal variation of carotene, vitamin A and computed total vitamin A activity of butter-fat.

The results in Table 1 show clearly the effect of pasture on the vitamin A potency of butter-fat and emphasize the cyclic nature of the variations mentioned by Koehn (1941). The steady decrease found by him between August and December was not observed. It would appear that so long as the cattle had access to pasture, even if for only a few hours a day, the potency of the butter was maintained. As soon as the cows were placed on a diet low in carotene, there was a marked decrease in the vitamin A activity of the butter, the potency in winter being only about half that in summer. This relative potency agrees with the findings of Berl & Peterson (1943), who obtained values of 22 and 40 i.u./g. of fat in winter and summer respectively. It is interesting to note that the carotene figures vary much more widely than do those for vitamin A *per se*. The carotene increases 4–5 times while the vitamin A is scarcely doubled. This observation agrees with those of Baumann & Steenbock (1933) and Sen & Sarkar (1943). It may be pointed out that the actual carotene figures, calculated as 94 % of the total yellow colour (Gillam, 1934) may be somewhat high. Koehn (1939) claims that only 91 % of the total colour is carotene, and other workers, for instance Zscheile, Nash, Henry & Green (1944) and Berl & Peterson (1943), have found only 75–80 % carotene in the total butter-fat carotenoids. Using the figures as they are, the contribution made by the carotene to the total vitamin A activity is 22–28 % during most of the year. This amount fell to 20 % or less during stall-feeding, and rose to the maximum of 35 % mentioned by Sen & Sarkar (1942) only during the first period of pasture-feeding in May and June.

*Serum*

The carotene and vitamin A content of samples of blood serum collected at approximately monthly intervals during 1944 and early 1945 are shown in Table 2. Carotene values are expressed as mg./100 ml. and vitamin A values as i.u./100 ml.

The maximum and minimum values both of carotene and of vitamin A agree well with those of Braun (1945), who obtained 1·35 and 0·3 mg./100 ml. for carotene and > 100 and 40 i.u./100 ml. for vitamin A. The results show no decrease while the cows have access to pasture, and in this respect, they differ from those of Braun who found a steady decrease from March to October. It would appear, therefore, that access to pasture even during limited

Table 2. *Carotene and vitamin A values in blood serum*

| | Carotene (mg./100 ml.) | Vitamin A (i.u./100 ml.) |
|---|---|---|
| August | 1·12 | 117 |
| September | 1·68 | 235 |
| October | 1·14 | 159 |
| November | 1·20 | 106 |
| December (beginning) | 1·44 | 173 |
| Stall-feeding started | | |
| December (end) | 0·38 | 60 |
| February | 0·26 | 25 |
| March | 0·14 | None found |
| April (beginning) | 0·13 | 40 |
| Pasture-feeding started | | |
| April (end) | 0·84 | 62 |
| May | 0·78 | 65 |
| June | 1·52 | 120 |

hours is enough to ensure adequate vitamin A nutrition as measured by the carotene and vitamin A of both serum and butter-fat. It has been pointed out that the seasonal variation of carotene in butter-fat is more marked than that of vitamin A *per se*. Examination of the serum results reveals that this effect is by no means so marked as with butter-fat. The average carotene content of serum from August to December is 1·32 mg./100 ml., and the corresponding vitamin A value is 158 i.u./100 ml. The corresponding averages for the winter months January to April are 0·23 mg. and 31 i.u. respectively. (This last average includes the possibly anomalous vitamin A result found in March.) In each case there is a 5–6-fold decrease during the winter months. When the cows were again put on pasture at the end of April the serum carotene increased at a greater rate than the vitamin A. This might mean that the liver reserves of vitamin A, depleted during stall-feeding, are first built up before the serum vitamin A increases again. If this is so, the results would seem to support the view of Boyer, Phillips, Lundquist, Jensen & Rupel (1942) who, from experiments on calves, suggested that measurement of serum vitamin A *per se* was a more sensitive criterion of vitamin A nutrition than was serum carotene. Finally, it may be mentioned that partly because of the small number of results available, and partly because they fall into two distinct groups instead of following a regular gradation, it is not possible to come to any conclusion about the use of the vitamin A/carotene ratio suggested by Braun as a means of assessing vitamin A nutrition.

## SUMMARY

1. Data are presented showing the seasonal variation over a period of 2 years of the vitamin A activity of butter-fat of Ayrshire cows. The average values for pasture- and stall-feeding were respectively 31 i.u. and 15 i.u./g. of fat.

2. The contribution made by carotene to the vitamin A activity of butter-fat ranged from 28% during pasture-feeding to 20% or less while the animals were in stalls. Carotene made its maximum contribution during May and June, accounting for 34% of the total vitamin A activity.

3. Serum carotene and vitamin A values during pasture-feeding were respectively 1·35 mg. and 150 i.u./100 ml. The corresponding values for stall-feeding were 0·23 mg. and 31 i.u./100 ml.

4. The results indicate that pasture-feeding even for only a few hours a day maintains high levels of carotene and vitamin A in both butter-fat and serum.

The author wishes to thank Prof. R. A. Morton for the use of spectroscopic apparatus, Dr H. Jasperson for encouragement and criticism and the Directors of J. Bibby and Sons, Ltd., for permission to publish this work.

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# A Modification of Current Micro-methods for the Estimation of Diodone (3:5-di-iodopyridone-*N*-acetic acid)

By J. A. BARCLAY and R. A. KENNEY, *Department of Physiology, University of Birmingham*

(*Received* 2 *July* 1945)

The investigation of renal function in health or disease demands a reliable method for the estimation of diodone in blood and urine. The methods of Alpert (1941), Flox, Pitesky & Alving (1942), Corcoran & Page (1943) all involve the use of bromine to split iodine from the diodone molecule and to convert this into iodate. Having had a fairly wide experience in the use of diodone in the estimation of renal blood flow and tubular mass in normal men and patients, as well as in animals, we have re-examined the various methods, and now present a procedure which, by a suitable combination of the best features of the other methods, combines accuracy with speed. By this method it is possible to perform the analysis of the blood and urine samples from a clearance test in about 30 min., whereas Alpert's method takes the greater part of a day. In the procedure here presented the diodone iodine is converted by a bromide-bromate mixture into iodate; the excess $Br_2$ removed by sodium formate and the iodate estimated colorimetrically by acidification and the addition of excess KI.

## METHOD

### *Reagents*

*Bromide-bromate mixture.* 10 g. $KBrO_3$ (A.R.) and 80 g. of anhydrous NaBr (A.R.) are dissolved in 100 ml. of distilled water, 40 ml. of 85% $H_3PO_4$ are added and the mixture shaken and allowed to stand for 24 hr. before use. The solution is stored in a glass-stoppered bottle and kept in the refrigerator when not in use.

*Sodium formate solution* 10%. 10 g. of the A.R. solid are made up to 100 ml. of solution with 80% ethanol.

*Alkaline potassium iodide.* 50 g. KI (A.R.) are made up to 100 ml. of solution with N-NaOH.

*Phosphoric acid* 85%. A.R. grade acid of specific gravity not less than 1·7.

### *Procedure*

2 ml. of a tungstic acid filtrate of plasma, or urine diluted to the plasma concentration of diodone, are placed in a $5 \times \frac{5}{8}$ in. hard-glass test-tube and 1 drop of the bromide-bromate mixture added. The tube is shaken until the solution is an even yellow, and then placed in a boiling water-bath for 3 min. It is removed, and, in order to speed up the procedure, cooled in iced water. 2 ml. of 10% Na-formate are added from a blow-out pipette in such a manner as to wash down the sides of the tube. The tube is shaken vigorously for a few seconds and then allowed to stand at laboratory temperature for 15 min. with occasional shaking. 2 drops (0·1 ml.) of 85% $H_3PO_4$ are added and the tube shaken until the *schlieren* figures are no longer visible in the solution. One drop (0·05 ml.) of alkaline KI is then added and the solution mixed. The colour is allowed to develop for 10 min. at laboratory temperature, at the end of which time it is estimated in a photoelectric colorimeter, using a filter of blue Chance glass (O.B.2). A calibration curve is prepared from aqueous solutions of diodone over the range of 0–10 mg. of diodone/100 ml.

It is essential to leave the tubes for 15 min. after the addition of the formate in order to ensure that all the residual $Br_2$ has been discharged. If any noticeable colour is developed in a blank estimation, each reagent should be checked, and, if necessary, made up afresh.

## RESULTS

The ideal method should allow a quantitative recovery of iodine from diodone. We performed the estimation on standard aqueous solutions of recrystallized 3:5-di-iodopyridone-*N*-acetic acid freed of diethanolamine and diethylamine, and in Table 1 we show the recovery by various methods, using as

Table 1. *Amount of diodone* (3:5-*di-iodopyridone-N-acetic acid*) *recovered from aqueous solutions by different methods*

| Amount of diodone present (mg./100 ml.) | Diodone recovered (mg./100 ml.) | | | | |
|---|---|---|---|---|---|
| | Composite method | Corcoran & Page | Flox, Pitesky & Alving | Colorimetric modification of Alpert's method | Composite method using oxalate instead of formate |
| 0 | 0 | 0·30 | 0·30 | 0·50 | 0 |
| 0·1 | 0·10 | 0·60 | 0·60 | 0·60 | 0·13 |
| 1·0 | 1·00 | 1·40 | 1·10 | 1·20 | 1·10 |
| 2·0 | 2·00 | 2·40 | 2·50 | 2·00 | 2·00 |
| 4·0 | 4·00 | 4·80 | 4·70 | 4·80 | 4·80 |
| 5·0 | 5·05 | 5·70 | — | 5·70 | 5·25 |
| 6·0 | 5·97 | 6·50 | 6·36 | — | 6·30 |
| 7·0 | 6·94 | — | 7·40 | 7·60 | 7·20 |
| 8·0 | 8·08 | 8·20 | — | — | 8·40 |
| 10·0 | 10·00 | 10·50 | 11·50 | 10·00 | 11·00 |

standard the colour developed by solutions of iodate containing the equivalent amount of iodine. Fig. 1 shows the recovery of the acid added to samples of normal human urine.

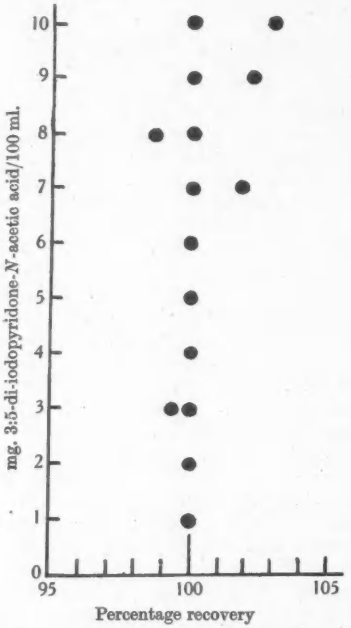


Fig. 1. Recovery of diodone (3:5-di-iodopyridone-*N*-acetic acid) added to normal human urine.

A point to be established is the best method of removing the bromine. Boiling it off, although apparently the most satisfactory method, does not, as our examination of Alpert's method shows, result in complete removal. Moreover, it is too tedious for routine use; the analyses of the urines and blood from one patient take about a day. Mass methods of boiling in a brine-bath lead to still more unsatisfactory results.

We have tried a number of reagents for this purpose and find that sodium formate is the best (Table 1). Oxalate is the only other substance that approaches it in efficiency. It should be noted that one or two of the other methods, if used on both blood and urine diluted to the same range, would give figures for clearances which would be reasonably accurate, since the blank value appears to be fairly constant.

Plasma is always liable to present difficulties. Occasionally a perfectly clear filtrate will, after addition of bromine and subsequent clearing, become quite cloudy. This cloudiness, due presumably to traces of protein, makes a colorimetric estimation impossible, and a titrimetric as well, since the bromine can never be completely removed under these conditions. Indeed the titrimetric application of Alpert's procedure has invariably given more inaccurate results than the colorimetric estimation when the filtrates are anything but perfectly clear. Titration of standard aqueous solutions treated by the Alpert method appears to give results slightly inferior to those obtained by colorimetry. The cloud is more usual in pathological material, but it sometimes occurs in normal human and animal plasma. We have therefore presented (Table 2) recoveries from various filtrates so that the effect of using a different protein precipitant can be assessed. It will be seen that the use of a detergent, suggested by Corcoran & Page (1943), increases the error, doubtless because its presence appears to give a much finer precipitate.

Table 2. *Amount of diodone (3:5-di-iodopyridone-N-acetic acid) recovered from normal human plasma deproteinized by various methods*

| Amount of diodone added (mg./100 ml.) | Diodone recovered (mg./100 ml.) Deproteinizing agent | | | |
|---|---|---|---|---|
| | Tungstic acid | Trichloro-acetic acid | Zinc hydroxide | Zinc hydroxide + detergent |
| 0 | 0 | 0 | 0·30 | 0·50 |
| 1·0 | 1·00 | 1·00 | 1·10 | 1·10 |
| 2·0 | 2·04 | 2·00 | 2·20 | 2·20 |
| 4·0 | 4·00 | 4·00 | 4·48 | 5·00 |
| 5·0 | 4·92 | 4·90 | 5·50 | 6·50 |
| 6·0 | 5·82 | 6·30 | — | — |
| 8·0 | 8·16 | 7·60 | 9·62 | 9·60 |
| 10·0 | 10·25 | 9·50 | 12·00 | 11·50 |

Since trichloroacetic acid filtrates have not in our experience turned cloudy, and since, as Table 2 shows, they give presentable recoveries, they may be used when difficulty is encountered with other filtrates.

## DISCUSSION

It appears almost certain that the amount of bromine present, whether as saturated bromine water (as used in the method of Corcoran & Page, 1943), or as a bromine mixture (Flox, Pitesky & Alving, 1942), is sufficient for the oxidation of the quantities of diodone iodine present, and, as the figures bear out, the real difficulty is in getting rid of the traces of residual bromine. In Alpert's method, where bromine is added directly, a fairly large residual blank is left, and perhaps accounts for the point he noted that the smallest amounts of diodone gave high readings. A bromide-bromate mixture is more satisfactory than a solution of bromine as it ensures that the amount of $Br_2$ added is precisely the same each time, which is unlikely with a bromine solution, even though it is in contact with liquid bromine.

Sendroy & Alving (1942) point out that increasing the amount of KI added to develop the colour increased the light absorption of the solution. Adding ethanol has the same effect. However, we find that this is not the case in the reaction used here; increasing the amount of KI or the concentration of acid or ethanol has no effect on light absorption, but the presence of ethanol increases the stability of the iodine colour, both in respect to time and to light. For this reason we have used an ethanolic solution of sodium formate as our clearing agent.

The ideal colour filter for photoelectric estimation of the iodine colour has been shown by Sendroy & Alving (1942) to be Corning glass Violet Ultra no. 586. This glass is not available in this country, and has the disadvantage of having so low a transmission as to require a very much more powerful source of light than is usual with photoelectric instruments. This latter condition also applies to the violet-blue Chance glass O.B.1 and the Wratten C filter, although, if the illumination of the instrument is adequate, one of these filters is to be preferred to the O.B.2 glass. We have, however, routinely used the Chance O.B.2 filter and have found it quite satisfactory and sufficiently sensitive over the range required.

## SUMMARY

Results of a study of the methods in current use for the estimation of diodone are presented together with details of a method incorporating the best features of previous methods.

We are indebted to Imperial Chemical Industries Limited for the supply of detergent.

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